

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification<sup>6</sup> :</b> <b>C12Q 1/68</b>		<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/55653</b> <b>(43) International Publication Date:</b> 10 December 1998 (10.12.98)
<b>(21) International Application Number:</b> PCT/US98/11457		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
<b>(22) International Filing Date:</b> 3 June 1998 (03.06.98)		<b>Published</b> <i>With international search report.</i>	
<b>(30) Priority Data:</b> 60/048,886 6 June 1997 (06.06.97) US 09/027,107 20 February 1998 (20.02.98) US			
<b>(71) Applicant:</b> NEXSTAR PHARMACEUTICALS, INC. [US/US]; Suite 200, 2860 Wilderness Place, Boulder, CO 80301 (US).			
<b>(72) Inventors:</b> CREIGHTON, Steven; 9799 E. Jewell Avenue #204, Denver, CO 80231 (US). GOLD, Larry; 1033 5th Street, Boulder, CO 80302 (US).			
<b>(74) Agents:</b> SWANSON, Barry, J. et al.; Swanson & Bratschun, L.L.C., Suite 200, 8400 E. Prentice Avenue, Englewood, CO 80111 (US).			

**(54) Title:** NUCLEIC ACID DETECTION**(57) Abstract**

A novel method for the highly selective detection of a specific target nucleic acid sequence in a sample composition that may contain a large number of nucleic acids. A copy of a target nucleic acid sequence is first formed by extension from a first primer complementary to part of the target sequence. A hybrid is then formed between this copy of the target nucleic acid sequence and a second primer, and the detection of the target nucleic acid sequence is based on the formation of pyrophosphate and/or dNMP.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LJ	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## NUCLEIC ACID DETECTION

5 This application claims priority under 35 USC § 119(e) from U.S. Provisional Patent Application No. 60/048,886, filed June 6, 1997.

### FIELD OF THE INVENTION

10 This invention is directed to a novel method for the highly selective detection of one or more specific target nucleic acid sequences in a sample composition that may contain a large number of nucleic acids. Included within the scope of this invention is a diagnostic kit for detecting the presence of target nucleic acid sequences in a sample composition that may contain such targets.

15 BACKGROUND OF THE INVENTION

The ability to detect the presence of a specific nucleic acid sequence in a composition or solution has proved to have increasing importance in a large number of applications. One of the most significant applications utilizing sensitive and selective detection of specific nucleic acid sequences is in diagnostic assays. The 20 development of diagnostic assays that rely on the ability to detect the presence of specific nucleic acids was, in great part, a result of the development of amplification-based nucleic acid detection schemes that allow for tremendous selectivity and specificity.

The polymerase chain reaction (PCR) was the first and remains the primary 25 amplification based nucleic acid detection scheme. Using natural enzymes and powerful amplification techniques, it is possible to identify the presence of a specific nucleic acid in a sea of diverse nucleic acids, even when only a very few copies of the sought after sequences are in the sample being tested.

Nucleic acid detection schemes (e.g., PCR, 3SR, SDA, Southern 30 hybridization) all rely on hybridization of one or more probes or primers which are complementary to a small portion (approximately 20 nucleotide residues) of the sequence of interest -- often a gene or mRNA but in principle any nucleic acid sequence. Hybridization of the probes to nearly complementary sequences -- which

-2-

would lead to false positives -- is suppressed by increasing the stringency of the hybridization. Stringency is typically controlled with temperature, although other variables, such as ionic strength, will affect stringency. The goal of a stringent hybridization protocol is to produce stable hybrids if and only if the target nucleic acid 5 is present in the media. Optimization of hybridization is a routine procedure in the art.

Following hybridization, nucleic acid detection schemes require a procedure to macroscopically register the presence of the hybrids. Direct methods like Southern hybridization use the presence of a tag (radioactive, enzymatic, fluorescent) affixed to the probe to signal the presence of a hybrid. Since un-hybridized probes constitute a 10 huge background, direct methods invariably require immobilizing the target to a solid phase so that un-hybridized probes can be washed away. Direct methods suffer from long assay times (up to 1 day) and relatively low sensitivity, with higher sensitivity coming at the cost of longer assays and the use of highly radioactive probes.

Recently developed indirect assays like PCR circumvent some of the 15 shortcomings of direct assays through the use of enzymatic amplification of the target sequence. Using several rounds of amplification it is possible to generate a large number of copies of the sequence of interest, which allows for highly specific and sensitive detection of the target nucleic acid. For example, the amplified target sequence can bind ethidium bromide (EtBr) and give a fluorescent signal. Since un- 20 hybridized probes do not bind EtBr and do not lead to amplified target, they do not provide a background signal and the assay can be run in the liquid phase. The assay in principle has no sensitivity problems since, theoretically, a single target sequence can be detected. However, as the technique involves multiple amplification steps, there is always a possibility of a contaminating sequence --that fortuitously bears some 25 sequence homology to the target sequence-- being produced. These methods also suffer from the need for complicated equipment, highly trained operators and moderately long assay times (about 4 hours).

PCR is initiated by the formation of hybrids between the target nucleic acid and synthetic complementary primers. A class of naturally occurring enzymes is then 30 used to "extend" the hybrid. At the 3' end of the primer, a recognizable feature is formed at the point where a double stranded nucleic acid is directly adjacent to a

single stranded region -- the rest of the target sequence. DNA polymerases are able to recognize this feature and, in the presence of the appropriate deoxy nucleotide triphosphate (dNTP) that is complementary to the first nucleic acid residue of the single stranded region, will link the appropriate complementary residue to the 3' end 5 of the primer. The DNA polymerase facilitated reaction creates the monophosphate link between the 3' end of the primer and the dNTP, thus releasing the inorganic species, pyrophosphate (PPi). So long as the appropriate dNTP is present, the chain will "grow" by the DNA polymerase catalyzed introduction of new residues. This 10 reaction, which occurs naturally, and the existence, characterization and purification of DNA polymerases have been known for many years.

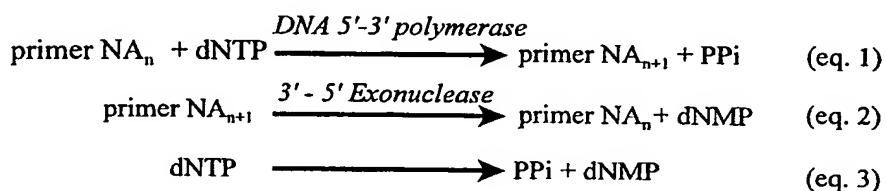
Also known to those skilled in the art is that most DNA polymerases have at least some 3'-5' exonuclease activity. 3'-5' exonuclease activity is the ability to cleave the 3' nucleic acid residue of a hybridized primer. This activity, therefore, reverses the reaction catalyzed by DNA 5'-3' polymerase activity by removing a 15 deoxy nucleotide-monophosphate (dNMP). The 3'-5' exonuclease acts at the same site recognized by the DNA polymerase. In some cases, the 3'-5' exonuclease domain exists on the same polypeptide chain as the DNA polymerase, but is a distinct domain; in other cases, the 3'-5' exonuclease is a separate polypeptide that is non-covalently associated with the polymerase. Certain DNA polymerases have enhanced 3'-5' 20 exonuclease activity while others -- naturally or by engineering -- have no ability to remove the 3' nucleotide. It is believed that utilizing a DNA polymerase with significant 3'-5' exonuclease activity allows for a reduction in misincorporation of nucleotides. Hence this activity is commonly referred to as 3'-5' proofreading exonuclease activity.

25 Systems can be engineered where a hybrid is formed between a primer and a template, and the composition of the dNTPs present is controlled in order to create a steady-state or "idling" condition. For example, if the first nucleic acid residue on the template 5' to the 3' terminus of the primer is an adenine, deoxy thymidine triphosphate (dTTP) must be present for the DNA polymerase reaction to proceed 30 (Equation 1). However, if the next 5' residue on the template is a guanidine and the solution does not contain any deoxy cytosine triphosphate (dCTP), an additional 3'

-4-

residue will not be added to the 3' end of the primer sequence. The 3'~5' proofreading exonuclease activity of the DNA polymerase will then remove the 3' terminal TMP incorporated by the successful DNA polymerase reaction (Equation 2). As long as dCTP is not introduced into the solution, a net reaction will occur as follows

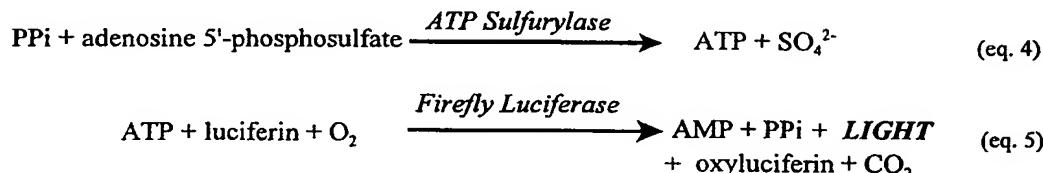
5 (Equation 3):



10

This reaction is also depicted in Figure 5. While idling, the hybrid-polymerase complex under these conditions converts dNTP into PPi and dNMP at a reaction rate of from 1/sec to 100/sec. Adjustment of the concentrations of reagents, and of the temperature of the reaction, will influence the rate of reaction.

15 There exist a number of systems for the ultra-sensitive detection of pyrophosphate (PPi). Some such detection systems rely on coupled enzyme reactions 20 that convert PPi into ATP, and then convert ATP into light using the firefly luciferin/luciferase system. One example of such a scheme is known as ELIDA. The reactions that take place in ELIDA are as follows.



25

The "firefly reaction", relying on the catalyzed oxidation reaction of luciferin (eq. 5),

-5-

is well known. The net of the reactions of Equation 4 and Equation 5 does not generate or consume PPi, but requires PPi in order to proceed. Because of the high sensitivity of luminescence detectors, ELIDA is therefore a sensitive assay for detecting the presence of pyrophosphate.

5 Another form of PPi detection uses the enzyme Nicotinamide Dinucleotide Pyrophosphorylase (NAD Phosphorylase). This enzyme catalyzes the formation of ATP from NAD and PPi. The ATP may then be detected using the luciferin/luciferase system described above.

Pal Nyrén and coworkers (Nyrén, *et al.*, *Anal. Biochem.* 208:171-175 (1993);  
10 Ronaghi *et al.*, *Anal. Biochem.* 242:84-89 (1996)) have developed an elegant scheme for sequencing nucleic acids taking advantage of the ELIDA assay. Termed Sequencing by Synthesis, a nucleic acid to be sequenced is immobilized on a solid support, and complexed with a complementary primer to form a hybrid species. The separate dNTPs are introduced to the solution containing the hybrids in the presence  
15 of a DNA polymerase. The release of pyrophosphate, detected by ELIDA, is an indication that the dNTP was complementary to the 5' residue on the target template. The intensity of the light output can be analyzed to determine when more than one identical sequence is found adjacent to another. Because it was necessary to quantify the light output of the assay, conditions were selected to minimize or eliminate idling.  
20 Thus, a DNA polymerase was selected that had no 3'-5' proofreading exonuclease activity. By contrast, the instant invention depends critically on the use of a DNA polymerase that does possess 3'-5' proofreading exonuclease activity.

#### BRIEF DESCRIPTION OF THE FIGURES

25 Figure 1 illustrates an overview of the central subject method. A Target Nucleic Acid in a Sample Composition (A) is hybridized under stringent conditions to one or more Probe Primers. At least one of the Probe Primers is conjugated to a group, such as biotin, that will allow that primer to be attached to a suitably functionalized Solid Phase Support (B). Following hybridization, the Target Nucleic  
30 Acid is partitioned from the Sample Composition by the addition of a Solid Phase Support that can bind to the conjugated Probe Primer. In this case, the Probe Primer

is biotinylated and the Solid Phase Support comprises streptavidin or avidin conjugated beads (C). Following isolation of the Solid Phase Support, a DNA Polymerase and a dNTP are added. The dNTP is the next nucleotide that will be added to the 3' end of the Probe Primer by a 5'→3' DNA Polymerase using the Target 5 Nucleic Acid as a template. The DNA Polymerase possesses 3'→5' Proofreading Exonuclease activity, and repeatedly incorporates and then excises the dNTP residue from the 3' terminus of the Probe Primer. The net result is the production of dNMP and PPi (C). In preferred embodiments, PPi is converted to NADH, and NADH is converted to light by the bacterial luciferase system.

10

Figure 2 illustrates the use of an Imprint of the Target Nucleic Acid. The Target Nucleic Acid is hybridized to an Imprint Primer (IP) that is labelled with a biotin group (B) to permit attachment of the Imprint Primer to a streptavidin-coated bead (S). The Imprint Primer is extended under stringent conditions to form a copy of 15 the Target Nucleic Acid. This copy may be formed from Nuclease Resistant Nucleic Acid Residues. If the Imprint is comprised of Nuclease Resistant Nucleic Acid Residues, then the Sample Composition may be degraded by nuclease treatment. In either case, the beads are then isolated from the Sample Composition. One or more Probing Primers are hybridized to the Imprint (sites P1-P3), and a dNTP and a DNA 20 Polymerase are added to establish Idling. The PPi produced by Idling is converted into NADH, and the NADH is consumed to generate light.

Figure 3 illustrates a Solid Phase Support-bound primer binding to a complementary nucleic acid.

25

Figure 4 illustrates a primer-target hybrid attached to a Solid Phase Support that is engaged and extended by a DNA polymerase.

30

Figure 5 illustrates the Idling phenomenon, and that the net result of Idling is the production of dNMP and PPi.

Figure 6 illustrates the different mass transport capabilities of bead columns and macroporous monolithic media.

5 Figure 7 depicts certain amplifications possible in the conversion of PPi to light using the NADH generating system and the bacterial luciferase system.

Figure 8 illustrates the detection of M13 template in the presence of a large excess of human genomic DNA according to the method of the invention.

10 Figure 9 depicts one embodiment of chemistry useful in attaching a nucleic acid to a Solid Phase Support, and also a cartridge-type macroporous monolith.

#### SUMMARY OF THE INVENTION

The present invention includes a method for detecting a target nucleic acid in a solution or composition. The method can also be used to detect the presence of multiple target nucleic acids simultaneously, and can be used to directly sequence the detected target nucleic acid. The methods of the subject invention will find utility in any application where it is necessary to detect the presence or absence of one or more specific nucleic acid sequences. Typical uses include, but are not limited to, detection of pathogenic organisms and viruses, diagnosis of genetic diseases, forensic analysis of bodily fluids, analysis of food substances, and environmental testing. Also included within the scope of this invention is a diagnostic kit that utilizes the method of the invention to detect a target nucleic acid in a composition that may contain such target.

25 According to the central method of this invention, at least a portion of the sequence of the target nucleic acid is known. One or more primers, complementary to sequences within the target nucleic acid are contacted with the sample composition so that hybrids are formed between the target and the primers. The sequences of the primers are chosen such that the identity of the nucleotide on the target that is immediately 5' to where the primers hybridize is known. Once the hybrid between the primer and the target has been formed, a DNA polymerase and the specific dNTP

complementary to the first residue of the target 5' to the hybrid is added to the solution.

In the preferred embodiment of the invention, the DNA polymerase is one that has substantial proofreading 3'-5' exonuclease activity. Therefore, in solutions where 5 hybrids have been formed -- indicating that the sample composition contains the target nucleic acid -- DNA polymerase will catalyze the conversion of dNTP to dNMP and pyrophosphate (PPi). If an idling state is reached, the concentration of dNMP and PPi will increase with time. Reaction conditions can be designed such that the production of PPi and dNMP depends linearly on time and the concentration of the hybrids in the 10 sample. In sample compositions not containing the target nucleic acid, there should be no generation of either dNMP or PPi.

According to the method of the invention, it is the "idling" of the DNA polymerase/Exonuclease reactions generating dNMP and PPi that provides "amplification" or "signal enhancement" of the hybridization event. Thus, even in a 15 sample composition containing only one copy of the target nucleic acid, given an idling rate of 500/min, after 10 minutes the amount of dNMP and PPi formed would be 5,000 molecules.

In preferred embodiments, the abovementioned steps are performed on a copy or "imprint" of the target nucleic acid. The copy is formed by first adding a primer to 20 the sample composition, wherein the primer hybridizes to a portion of the target. The primer is then extended in the presence of a DNA polymerase and dNTPs to form a copy of the region of the target nucleic acid where the idling reactions will take place. In these embodiments, the primer used to make the imprint may include some tag which will allow the imprint to be isolated from the reaction mixture. For example, 25 the primer may be biotinylated to allow the imprint to be attached to a streptavidin-coated solid phase support. In this way, the imprint may be isolated from the sample composition prior to establishing idling conditions. This greatly reduces the background signal produced by contaminating non-target nucleic acids. The imprint may optionally be comprised wholly or partly of nuclease resistant nucleic acid residues, and then treated with nucleases. This reduces the background even further 30 as only the imprint will survive nuclease treatment.

-9-

The method of this invention also includes means for sensitively measuring the generation of dNMP or PPi to indicate the presence of the target nucleic acid in the sample composition. Any method for detecting PPi and/or dNMP is contemplated in the instant invention. In some embodiments, the generation of PPi is detected to give 5 rise to a positive reading. In preferred embodiments, the generation of PPi is measured by an enzymatic system that first converts PPi to NADH, and then consumes the NADH to generate light via the bacterial luciferase system. This system uses a well known series of enzymatically-catalysed reactions to convert PPi to NADH.

10 The NADH produced can be assayed in a variety of ways well known in the art. Firstly, NADH can be assayed in the bacterial luciferase bioluminescence system that is well known in the art; this is the preferred method of NADH detection in the invention. Secondly, the NADH can be detected directly through fluorescent assays. Thirdly, NADH can be detected by electrochemical assays. Additionally, NADH can 15 be assayed by a variety of colorimetric assays known in the art.

PPi generation can also be measured by converting adenosine 5' phosphosulfate to adenosine triphosphate (ATP) in the presence of the enzyme ATP sulfurylase. Alternatively, PPi generation may be measured by converting 20 nicotinamide dinucleotide into nicotinamide mononucleotide, with the concomitant generation of ATP, in the presence of NAD pyrophosphorylase. In both cases, the ATP thus formed is combined with firefly luciferin in the presence of the firefly enzyme luciferase, and light is generated. In these cases, the dNTPs in the DNA Polymerase reaction mixture must be removed prior to detecting the ATP, as all 25 dNTPs can activate the firefly luciferase to some extent.

25

#### DETAILED DESCRIPTION OF THE INVENTION

This invention includes a novel method for the detection of a target nucleic acid sequence. The basis for the detection scheme of this invention is the formation of a hybrid between the target nucleic acid sequence and a primer sequence 30 complementary to the target. The presence of the hybrid is detected based on the formation of pyrophosphate (PPi) and/or deoxy nucleoside monophosphate (dNMP) in

-10-

the presence of a DNA polymerase and the appropriate deoxy nucleoside triphosphate (dNTP). By appropriately adjusting the operating conditions of the solution, each hybrid can generate detectable amounts of PPi and/or dNMP. In the preferred embodiment of the invention, the detection of hybrid formation -- and thus the 5 detection of the target nucleic acid sequence -- is accomplished by detecting the presence of PPi. Further in the preferred embodiment, the presence of PPi is detected by assays that rely on a well known enzyme system that converts PPi to NADH. The NADH is then consumed to generate light using the bacterial luciferase system. This detection system has the sensitivity to detect as few as 100 PPi  
10 molecules in a sample. The methods described herein have the potential to detect a single copy of a target nucleic acid.

In preferred embodiments, the idling conditions are established on a copy of the target nucleic acid that is synthesized in the sample composition, and then attached to a solid phase support. Creating this copy, which can be isolated from the sample 15 composition by virtue of its association with a solid support, greatly enhances the sensitivity of the central method.

Various terms are used herein to refer to aspects of the present invention. To aid in the clarification of the description of the components of this invention, the following definitions are provided:

20 "Nucleic Acid" as defined herein refers to oligonucleotides including either DNA, RNA, single-stranded or double-stranded and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid bases or the nucleic acid  
25 as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil, backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like.  
30 Modifications can also include 3' and 5' modifications such as capping.

-11-

"Target Nucleic Acid" as defined herein refers to any nucleic acid that it is desired to be detected by the present invention. In preferred embodiments of the invention, the Target Nucleic Acid is genomic DNA, genomic RNA or a specific mRNA. In certain embodiments of the invention, the presence of the Target Nucleic Acid in a sample composition is a marker for a specific disease or medical condition. For example, markers to diseases are known in D53 and sas mutations and for sickle cell anemia.

"Sample Composition" as defined herein refers to any heterogenous or homogenous solution being tested for the presence of the Target Nucleic Acid. The Sample Composition may be a biological fluid (i.e., blood, serum, urine etc.) or it may be any prepared solution that may contain the Target Nucleic Acid. In addition to the Target Nucleic Acid, the Sample Composition may contain other nucleic acids, as well as any other components, including, but not limited to, proteins, peptides, carbohydrates and any other components, so long as the components of the Sample Composition do not interrupt the ability of the Primer to hybridize with the Target Nucleic Acid. In certain embodiments of the invention, certain characteristics of the Sample Composition (i.e., pH, temperature, ionic strength) must be adjusted in order to allow conditions for hybrid formation to occur. The manipulation of such conditions is well known to those skilled in the art.

"Probe Primer" as defined herein refers to a nucleic acid sequence that is complementary to at least a portion of the Target Nucleic Acid. The Probe Primer is comprised of Nucleic Acid residues, and may be from 3-100 nucleotides long. In the preferred embodiment, the Primer is about 6-30 nucleotides long. In some embodiments, the Probe Primer is comprised in whole or in part of Nuclease Resistant Nucleic Acid Residues and/or Thermostable Nucleic Acid Residues. The use of Thermostable Nucleic Acid Residues, such as PNA, increases the ability to eliminate mismatches between the Probe Primer and nucleic acid sequences similar but not identical to those found in the Target Nucleic Acid. In preferred embodiments, the Probe Primer is used to establish Idling on an Imprint copy of the Target Nucleic Acid. In other embodiments, the Probe Primer includes a tag which allows the Probe Primer, and the Target Nucleic Acid that is hybridized thereto, to be removed from the

reaction medium. For example, the Probe Primer may be biotin-labelled and used to establish Idling directly on a Target Nucleic Acid while the Probe Primer is associated with a streptavidin-coated Solid Phase Support. "Imprint Primer" as defined herein refers to a nucleic acid sequence that is complementary to at least a portion of the

5 Target Nucleic Acid 3' to where the Probe Primers Hybridize. The Imprint Primer is comprised of nucleic acids, and may be from 3-100 nucleotides long. The Imprint Primer may be comprised wholly or partly of Nuclease Resistant Nucleic Acid Residues and/or Thermostable Nucleic Acid Residues. The use of Thermostable Nucleic Acid Residues, such as PNA, increases the ability to eliminate mismatches

10 between the Primer and nucleic acid sequences similar but not identical to those found in the Target Nucleic Acid. In the preferred embodiment, the Imprint Primer is about 6-30 nucleotides long. In preferred embodiments of the invention, the Imprint Primer is labelled at one or more sites with molecules that permit attachment to a Solid Support. Such attachment can be accomplished by a number of methods known to

15 those skilled in the art. In one preferred embodiment, the Imprint Primer is biotin-labeled, and will bind to a streptavidin-coated Solid Support.

"Imprint" as defined herein refers to a copy of the Target Nucleic Acid made by hybridizing an Imprint Primer to a Target Nucleic Acid in the Sample Composition, followed by extending the 3' end of the hybridized Imprint Primer with Nucleic Acid residues and a DNA Polymerase. The Imprint contains the sequences that will be hybridized to the Probe Primers. In preferred embodiments, the Imprint Primer is extended with Nuclease Resistant Nucleic Acid Residues to form a nuclease resistant Imprint. The Imprint may also be comprised wholly or partly of Thermostable Nucleic Acid Residues. The fidelity of Imprint formation can be increased by any of the methods well known in the art. These methods increase the specificity of Nucleic Acid hybridization, and thereby prevent the formation of non-Target Nucleic Acid Imprints through mispriming. Such events can be avoided by performing hybridization at elevated temperatures and adjusting the ionic strength of the hybridization reaction. Suitable methods contemplated in preferred embodiments involve the use of thermostable DNA Polymerases, such as *Taq*, at high temperatures. In other embodiments, fidelity is further improved through the inclusion of single

-13-

strand DNA binding proteins, such as T4 gp32 and *E. coli* SSB, in the reaction mixture used to form the Imprint. In still further embodiments, betaine ((CH<sub>3</sub>)<sub>3</sub>N<sup>+</sup>-CH<sub>2</sub>-COO<sup>-</sup>) can be included in order to promote the isothermal denaturation of Nucleic Acids in the Sample Composition. This will prevent GC rich Target Nucleic Acids from being 5 relatively poorly denatured prior to addition of the Imprint Primers.

"DNA Polymerase" as defined herein refers to a family of enzymes known to those skilled in the art. DNA Polymerases are enzymes that recognize the junction between single-stranded and double-stranded nucleic acids created by the hybridization of primer to a Target Nucleic Acid. DNA Polymerases useful in the 10 present invention include, but are not limited to, T4 DNA Polymerase, T7 DNA Polymerase, T7 DNA Polymerase:Thioredoxin, thermostable DNA Polymerase from *Pyrococcus woesei*, Klenow Fragment DNA Polymerase, AMV Reverse Transcriptase and MMLV Reverse Transcriptase Polymerase. Preferred DNA Polymerases have 3'- 15 5' Proofreading Exonuclease activity. The preferred DNA Polymerases of the present invention are T4 DNA Polymerase, T7 DNA Polymerase, T7 DNA Polymerase:Thioredoxin, and the thermostable DNA Polymerase from *Pyrococcus woesei*.

Various reagents can be included in the buffer containing the DNA Polymerase in order to stabilize the enzyme or maximize its activity. For example, the addition of 20 co-solvents can act to stabilize the activity of DNA Polymerases at elevated temperatures. In one such embodiment, the disaccharide trehalose is used to thermostabilize or thermoactivate DNA Polymerases that are normally inactive at elevated temperatures (Carnici *et al.* 1998. Proc. Natl. Acad. Sci. 95: 520-524; incorporated specifically by reference herein). In the presence of trehalose, AMV 25 Reverse Transcriptase can function efficiently at 60°C. Additionally, the osmolyte betaine is known to confer thermostability to a number of proteins.

"Solid Phase Support" as defined herein refers to any of a number of supports compatible with the reagents of the present invention. Solid Phase Supports can take the form of beads, filters, plugs or plates. Solid Phase Supports are typically made of 30 inert materials that are functionalized on their surface to allow for the attachment of Primers. Preferred Solid Phase Supports include beads, Macroporous Supports, and

-14-

microfabricated planar surfaces, commonly known in the art as "biochips". In particularly preferred embodiments of the invention, the Solid Phase Support is a streptavidin-coated paramagnetic bead that can be isolated from reagent solutions by the application of a magnetic field.

5 "Thermostable Nucleic Acid Residues" as defined herein refers to modified nucleic acid residues that are stable under increased temperature conditions. Examples of Thermostable Nucleic Acid Residues are 2'F RNA and PNA.

10 "Nuclease Resistant Nucleic Acid Residues" as defined herein refers to modified nucleic acid residues that are stable in the presence of enzymes with nuclease activity. Enzymes with nuclease activity include DNase I and Exo III, and certain DNA Polymerases. The use of any Nuclease Resistant Nucleic Acid Residue is contemplated in the instant invention. In one preferred embodiment, the Nuclease Resistant Nucleic Acid Residues are phosphorothioate Nucleic Acid residues (dNMP $\alpha$ S). The Imprint is made to contain dNMP $\alpha$ S by giving the Polymerases 15 which copy the initial Sample Composition dNTP $\alpha$ S instead of dNTP. Most Polymerases will accept this substitution without loss of efficiency and fidelity.

20 "Macroporous Supports" as defined herein refers to a class of Solid Phase Support materials characterized by a permanent porous structure formed during their preparation that persists in the dry state. These materials are comprised of cross-linked polymers (See, F. Svec *et al.*, *Science* 273:205 (1996), incorporated herein by reference).

25 "PPi Detection Assay" as defined herein refers to any assay or method for detecting the formation or presence of pyrophosphate (PPi) in an aqueous solution. PPi can be detected by a variety of analytical methods, including, but not limited to: 1) luminescent, 2) fluorescent, 3) colorimetric, 4) light absorption, and 5) electrochemical. Any enzymatic system that can convert PPi into light is included within the scope of the subject invention. The preferred method of PPi detection uses a coupled series of enzyme reactions to convert PPi into NADH; the NADH is then consumed to generate light in preferred embodiments. In other embodiments, the 30 NADH is detected through electrochemical assays, colorimetric assays, or fluorometric assays known in the art.

Other examples of sensitive PPi Detection Assays involve the use of enzymes that catalyze the conversion of PPi to ATP, followed by the generation of light from the ATP using the firefly luciferin/luciferase assay well known in the art. One example of such a coupled assay is ELIDA (See, Nyren *et al. Analytical Biochem.*

5 208:17 (1993), incorporated herein by reference). Still further examples include, but are not limited to, the NAD pyrophosphorylase-catalyzed reaction of PPi and NAD to form NADH and ATP, the adenylate-cyclase-catalyzed reaction of PPi and cyclic AMP to form ATP, the ADP-Glucose Pyrophosphorylase-catalyzed reaction of ADP-glucose and PPi to form ATP and 1-phosphoglucose, and the poly (A) polymerase-10 catalyzed reaction of Poly (A)<sub>n</sub> and PPi to form Poly (A)<sub>n+1</sub> and ATP.

Also included is a spectrophotometric method that is a coupled assay in which the addition of inorganic pyrophosphatase initially cleaves the pyrophosphate into two molecules of phosphate, and the phosphate is then detected by the conversion of 2-amino G-mercapto-7-methyl prime ribonucleoside to 2-amino-G-mercapto-7-methyl prime by prime nucleoside phosphorylase (See, Upson *et al. Analytical Biochem.* 15 243:41 (1996), incorporated herein by reference).

"Idling" as defined herein refers to a coupled enzymatic process wherein DNA Polymerase catalyzes the linkage of a dNTP to a DNA at a point of hybridization to generate PPi, while the 3'→5' Proofreading Exonuclease activity of the DNA 20 Polymerase removes the linked dNMP. The net reaction when a hybrid system is Idling is the conversion of dNTP to dNMP and PPi. Idling can occur indefinitely if the appropriate dNTP concentration is maintained in solution and the PPi product is removed. Idling only occurs when a hybrid is formed between the Probe Primer and the Target Nucleic Acid. In order to maintain idling, the complementary dNTP 5' to 25 the end of the newly formed linkage is not included in the solution. Idling will only occur when the DNA Polymerase has 3'→5' Exonuclease activity.

"3'→5' Exonuclease Activity" and "3'→5' Proofreading Exonuclease Activity" as defined herein refers to the ability to catalyze the cleavage of the last 5' nucleic acid residue of the Primer that is part of a hybrid formed between the Primer and the Target 30 Nucleic Acid sequence. Certain DNA polymerases have enhanced 3'→5' Exonuclease activity. Typically DNA 5'→3'polymerase activity and 3'→5' Exonuclease Activity are

found as distinct domains of the same polypeptide on the same gene. For example, E. coli pol I and T4 DNA Polymerases contain 5'-3' polymerase and 3'-5' Exonuclease Activity on a single polypeptide chain.

The present invention for detecting the presence of a Target Nucleic Acid in a Sample Composition involves the combination of a number of known principles and reactions. The basic steps of the method include 1) formation of a hybrid between the Target Nucleic Acid and the Probe Primer; 2) addition of DNA Polymerase reagents and establishment of Idling conditions in which PPi and dNMPs are produced if the hybrid is formed; and 3) detection of either or both of PPi and dNMP. The basic method is depicted in Figure 1, and the individual steps are described below in detail:

A) Contacting the Sample Composition with a Probe Primer that is complementary to at least a portion of the sequence of said Target Nucleic Acid.

The contacting step is conducted under conditions where a hybrid is formed between the Primer and Target Nucleic Acid if the Target Nucleic Acid is present in the Sample composition (Figure 1A). The conditions for hybridization are chosen so that the maximum possible stringency is achieved. Preferred embodiments use high temperatures, particular ionic strengths, and judicious choice of Probe Primer sequences to achieve high stringency. These factors are well known in the art, and are described in "Nucleic Acid Analysis", Ed. C. Dangler, Wiley-Liss 1997, the contents of which are specifically incorporated herein by reference. Any method known in the art for enhancing the stringency of Nucleic Acid hybridization is contemplated. For example, isothermal denaturation can be promoted by the addition of betaine.

In preferred embodiments of the invention, the Probe Primer is attached to a Solid Phase Support in order to facilitate the separation of hybrids that have been formed from the remainder of the Sample Composition (Figure 1B). Once attached to the Solid Phase Support, the hybrid is easily partitioned from the non-Target Nucleic Acids in the Sample Composition by any of the methods known in the art. In preferred embodiments, the Probe Primer is labeled with one or more biotin groups. The biotin-labelled hybrid may then be attached to a streptavidin-coated Solid Phase

Support, preferably a superparamagnetic bead. Such beads are commercially available (e.g., DYNABEADS® from Dynal Inc.) As both hybrids and free Probe Primer bind to a streptavidin-coated Solid Phase Support, the number of biotin-binding sites on the Solid Phase Support is in excess of the number of Probe Primers initially added to the 5 Sample Composition. If the Solid Phase Support comprises paramagnetic beads, then these can be separated from the Sample Composition by the application of a magnetic field.

Further in the preferred embodiments, the Probe Primer is partly or wholly comprised of Nuclease Resistant Nucleic Acid Residues. This prevents the Probe 10 Primer from being digested by the 3'→5' Exonuclease Activity of the DNA Polymerase during Idling.

In some embodiments of the invention, the Probe Primer comprises Thermostable Nucleic Acid residues. In these embodiments, the Probe Primer may include Thermostable Nucleic Acid residues at the 5' end of the Probe Primer and 15 regular DNA at its 3' end. This allows hybridization based on the Thermostable Nucleic Acid residues and, if the 3' end of the Probe Primer is the absolute minimum amount of DNA needed for Idling, the stable hybrids that survive a heating step will provide a link for the adjacent DNA-DNA duplex. Thus a very long sequence can be obtained to assure nearly absolute specificity.

20

B) Extending the Probe Primer by the addition of at least one dNTP and at least one DNA polymerase, wherein pyrophosphate and dNMP is produced by Idling.

In embodiments of the present invention where the Probe Primer is attached to 25 a Solid Phase Support, the hybrids formed between the Probe Primer and the Target Nucleic Acid are physically segregated from the remainder of the Sample Composition and washed to remove all components of the Sample Composition that do not bind to the Probe Primer (Figure 1C). In one embodiment of the invention, the trapped hybrids are cleaved from the Solid Support before the addition of the 30 appropriate dNTP and the DNA Polymerase that will catalyse the Idling reaction. The DNA Polymerase catalyzes the template-dependent 5'→3' extension of the Probe

Primer. The dNTP that is added is the nucleotide that will be the next one to be incorporated at the 3' terminus of the Probe Primer using the Target Nucleic Acid as a template.

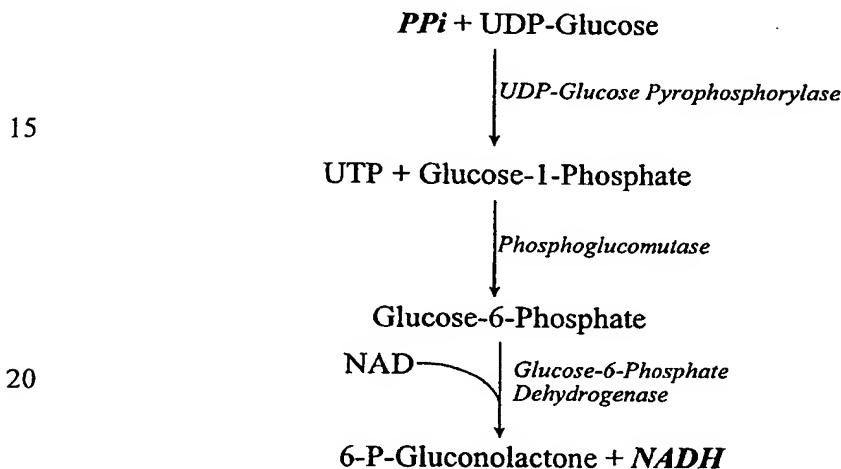
The DNA Polymerase used in the Idling reaction is one with a substantial 3'-5' Proofreading Exonuclease Activity in order that the dNTP is repeatedly incorporated and excised. Preferred DNA Polymerases include T4 DNA Polymerase, T7 DNA Polymerase: Thioredoxin and the thermostable DNA Polymerase from *Pyrococcus woesei*. In some embodiments, Idling is carried out at elevated temperatures in order to maximize the rate of PPi turnover. In the case of T4 DNA Polymerase, a 10 fold increase in the rate of Idling can be achieved by raising the temperature from 20°C to 10 45°C. In some cases, the stability of the enzyme at high temperatures can be enhanced by the addition of co-solvents, such as trehalose.

In another embodiment of the invention, the Probe Primer used to form hybrids with the Target Nucleic Acid need not be the hybrid site for DNA polymerase action. For example, following hybridization and separation of hybridized Target Nucleic Acid from the remainder of the Sample Composition, the hybrid could be dissociated. One or more additional Probe Primers complementary to the Target Nucleic Acid can then be added to the solution before establishing Idling conditions. In those embodiments of the invention where the initial hybrid is not dissociated prior to Idling, it is also possible to add additional Probe Primers into the solution so that Idling can occur at multiple sites on the Target Nucleic Acid. In both cases, this provides an additive means for amplifying the original single hybridization event. Thus, if ten Probe Primers to the Target Nucleic Acid were hybridized to the Target, and then Idling was established at each site, the amount of PPi and dNMP formed 25 would be increased by an order of magnitude.

C) Detecting the Presence of the PPi or dNMP Produced during Idling.

Following Idling for a predetermined time, the Idling reaction is quenched by either heat or EDTA, and the amount of PPi and/or dNMP is quantitated. The 30 presence of either of these molecules indicates the presence of the Target Nucleic Acid in the Sample Composition. In the preferred embodiments of the invention, the

formation of pyrophosphate is used to detect the hybrid formation event. The presence of pyrophosphate can be detected by a PPi Detection Assay. The preferred PPi Detection Assay of the present invention uses a series of enzyme reactions to convert PPi into NADH; the NADH is then consumed by bacterial luciferase to generate light. In these embodiments, the PPi is converted into NADH by addition of an appropriate amount of UDP-Glucose and NAD in the presence of the enzymes UDP-Glucose Pyrophosphorylase, Phosphoglucomutase, and Glucose-6-Phosphate Dehydrogenase to form NADH and 6-P-Gluconolactone. These reagents may be added during Idling, or after Idling is complete. The following series of reactions takes place in this scheme if PPi is present: (PPi=Uridine-5'-diphosphate, UTP=Uridine-5'-triphosphate):

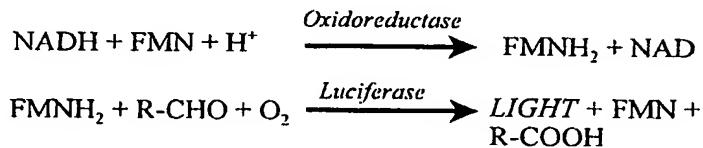


In preferred embodiments, the NADH produced in this system is then converted into light by using the bacterial luciferase system, well known in the art. This coupled enzyme system comprises an oxidoreductase which catalyzes the reduction of FMN by NADH, and a luciferase which catalyzes the reaction of the resulting FMNH<sub>2</sub> and a long-chain aldehyde to form a carboxylic acid with the concomitant emission of light at 495nm. The system is described by Hastings J., In: *Methods in Enzymology* New York, Academic Press 1978, 125-135, and by Jablonski, E. and DeLuca, M. In: *Clinical and Biochemical Luminescence*, New York, Marcel Dekker, 1982, 75-87, both of which are specifically incorporated herein by reference. The oxidoreductase

-20-

and luciferase that form the system can be isolated from a number of marine bacteria, including, but not limited to, *Lucibacterium harveyi*, *Beneckea harveyi*, *Photobacterium fischerii* and *Vibrio fischerii*. The reactions can be represented as follows (R-CHO = aldehyde; R-COOH = carboxylic acid):

5



10

The quantum yield of the reaction is between 0.2 and 0.27. The choice of aldehyde depends on the specific species of luciferase that is being used, but for *Photobacterium fischerii* the best intensity is achieved using decanal. As bacterial luciferases are not activated by dNTPs, there is no need to perform additional steps to 15 remove these nucleotides from the reaction mixtures. Thus, this method of detecting PPi is superior to prior art methods involving the use of ATP generating systems and the firefly luciferase system. Moreover, the assay conditions used in converting PPi to NADH are compatible with the all the various DNA Polymerase reagents.

Although preferred embodiments use the bacterial luciferase system, any 20 method for determining NADH is contemplated in the subject application. For example, there are techniques known in the art for detecting NADH by electrochemical assays, colorimetric assays, and fluorometric assays. In particular, the fluorometric assays provide a very sensitive means for assaying NADH. They rely on the ability of NADH to emit 420nm blue light upon excitation with 320nm ultraviolet 25 light. Techniques known in the art that use laser excitation have the capability of detecting single molecules of NADH in this way.

In other embodiments, the presence of PPi is detected by using an ATP generating system to convert the PPi to ATP, followed by the conversion of the ATP to light by the firefly luciferin/lucifase system. One example of such a system is 30 known as ELIDA. Another system uses the enzyme NAD-Pyrophosphorylase to catalyse the conversion of PPi and NAD to ATP and NMN. In these embodiments,

the mixture containing the Idling nucleic acid may be treated to remove unincorporated dNTPs prior to use of the PPi Detection Assay. Such unincorporated dNTPs can activate the firefly luciferin/luciferase system, albeit at a far reduced level compared to ATP. In some embodiments, the removal of dNTPs may be

5       accomplished by adding an appropriate amount of glycerol, glucose, and the enzymes glycerokinase and hexokinase. These enzymes can rapidly transfer the terminal phosphate groups of the dNTPs to glycerol and glucose, resulting in the formation of dNDPs that will not activate firefly luciferase. In other embodiments, the sequence of the Probe Primer(s) is chosen, if possible, such that dATP is the only dNTP that must

10      be added to establish Idling at each Probe Primer hybridization. Following the Idling reaction, dATP can be efficiently removed from the reaction mixture by adding the enzyme apyrase. In this embodiment, the other dNTPs (dGTP, dCTP, and dTTP) are not present at any time in the Idling reaction mixture.

In one embodiment of the invention, the PPi detection occurs in a separate vessel from the reaction chamber where the Idling reactions take place. In particularly preferred embodiments, the amount of PPi is quantitated by first converting all of the PPi in the quenched Idling reaction into NADH by means of the NADH-generating enzymatic systems described above. For example, in one embodiment, all of the PPi is converted to NADH by the addition of the necessary enzymes and reagents. Then, a

20      predetermined amount of the bacterial luciferase reaction mixture is injected into the quenched Idling reaction in front of a luminometer. The sudden oxidoreductase/luciferase-catalyzed reaction of all of the NADH results in the delivery of a "burst" of light which can be integrated for a period of time, typically 5 seconds, after injection. Systems that can perform this procedure automatically using multiwell plates are well

25      known in the art. The luciferase mixture in this embodiment preferably contains a large excess of the luciferase enzyme as the removal of the reaction products from the active sites of this enzyme is the rate-limiting step in light production. If the light signal is of sufficient size, then it is possible to document the signal on radiographic film.

30       The foregoing embodiments all involve the establishment of Idling conditions using a hybrid formed between the Target Nucleic Acid and one or more Probe

Primers. By using, for example, a biotin-labelled Probe Primer, it is possible to isolate the hybrids from the Sample Composition prior to performing the Idling reactions (Figure 1). This can be done, for example, by attaching the hybrids to streptavidin-conjugated paramagnetic beads, and then partitioning the beads through the application of a magnetic field. This isolation, although an optional addition to the central method, can markedly reduce the possibility of non-Target Nucleic Acids participating in the Idling reactions (e.g., intramolecular loops that act as self-priming templates for Idling), and will allow a degree of sensitivity and background discrimination to be attained that will be necessary for many applications. It is possible, however, to achieve further increases in the specificity of the technique through the performance of steps that involve the synthesis of a copy, or Imprint, of the Target Nucleic Acid. In these embodiments, the Sample Composition is first denatured and then contacted with an Imprint Primer. The Imprint Primer may be wholly or partly comprised of Nuclease Resistant Nucleic Acid Residues, and is labelled with a group (e.g., biotin) that permits subsequent attachment to a suitably conjugated Solid Phase Support. The Imprint Primer may also be comprised wholly or partly of Thermostable Nucleic Acid Residues. The Imprint Primer sequence is chosen so that it hybridizes to the Target Nucleic Acid at a position that is 3' to the location of the sequences that will later be used to establish Idling. A DNA Polymerase, dNTP molecules, dNTP $\alpha$ S molecules, Thermostable Nucleic Acid Residues, or combinations thereof are then added in order to form the Imprint by extension from the Imprint Primer. Agents that enhance the fidelity of DNA synthesis, such as single stranded binding proteins may also be included in the reaction mixture. The Imprint thus formed is labelled with biotin, for example, (due to inclusion of the biotinylated Imprint Primer) and comprises the sequences that will subsequently be recognized by the Probe Primer(s). In particularly favoured embodiments, the Imprint is formed from Nuclease Resistant Nucleic Acid residues using *Taq* DNA Polymerase at temperatures around 70°C.

Following Imprint formation, the Imprint is attached to a Solid Phase Support, and partitioned from the Sample Composition. In preferred embodiments this is achieved, as above, by the use of streptavidin-conjugated paramagnetic beads. The

isolated Solid Phase Support with attached Imprint is washed to remove any contaminating nucleic acids. In preferred embodiments, the washing is done under alkaline denaturing conditions in order to remove any non-specifically bound Nucleic Acids or nucleic acids that have hybridized to the Imprint, and to hydrolyze any 5 contaminating ribonucleic acid molecules. In order to further remove any contaminating non-Target Nucleic Acids, the washing may be optionally preceded or followed by the addition of nucleases, such as DNase I and Exo III. Nuclease treatment will further reduce the potential for contamination from non-Target Nucleic Acids, as only the Imprint Target Nucleic Acid is comprised of Nuclease Resistant 10 Nucleic Acid Residues and is spared from the action of the nucleases.

The isolated Imprint is then contacted with one or more Probe Primers, and Idling is established as described above. The Idling reactions can be performed with the Imprint attached to the Solid Phase Support, or the Imprint may first be detached therefrom.

15 In other embodiments, multiple rounds of Imprint formation can be used to achieve, if necessary, an even higher degree of specificity. Following the formation and partitioning of a first Imprint, the Imprint can be dissociated from the Solid Phase Support. A second biotinylated Imprint Prime -- which recognizes a sequence distinct from that recognized by the first Imprint Primer -- can then be added to the solution 20 containing the first Imprint, and a second Imprint can be formed and isolated as described above. This process can be repeated if necessary.

It will be appreciated from the foregoing that the creation of a Solid Phase 25 Support-bound Imprint can serve to drastically reduce the complexity of the Sample Composition in terms of the number of different nucleic acids molecules contained therein. Using the well established methods for performing stringent primer hybridization and extension during PCR, it is possible to remove essentially all non-Target Nucleic Acids from the sample solution. The only Nucleic Acids that remain are the Imprints of the Target Nucleic Acid. The foregoing embodiments therefore 30 enable one to optimize conditions in order to obtain the lowest background signal in the subsequent phases of the technique. Furthermore, the use of Nuclease Resistant Nucleic Acid Residues in both the Imprint and the Probe Primer will permit the use of

DNA Polymerases with substantial 3'-5' Proofreading Exonuclease Activity without concern that the Imprint or Probe Primer will be digested.

The use of Imprint and Probe Primers that recognize distinct sequences adds an additional degree of specificity to the method. If a non-Target Nucleic Acid in the 5 Sample Composition is fortuitously converted into an Imprint, due to some limited sequence homology with the true Target Nucleic Acid in the region used for Imprinting, then it will be highly unlikely that this Imprint will also contain the additional sites recognised by the Probe Primers. Therefore, only the true Imprint will serve as a template for Idling. Furthermore, the length of any mis-copied Imprints can 10 be kept to a minimum by allowing the Imprint formation polymerization reaction to proceed only for the minimum amount of time needed to copy the sequences recognized by the Probe Primers. This time can be easily determined by considering the polymerization rate of the DNA Polymerase used to form the Imprint (typically around 1000 base pairs/ minute). Keeping the length of any mis-copied Imprints to a 15 minimum further reduces the chance that the mis-copy will fortuitously contain a site for Probe Primer hybridization.

In alternate embodiments of the present invention, it is not necessary for Idling to be established in order to detect the presence of the hybrid formed between the Probe Primer and the Target Nucleic Acid or Imprint. In this embodiment, the hybrid 20 containing solution -- which can be but does not necessarily have to be separated from the remainder of Sample Composition -- is adjusted to contain a DNA Polymerase and all of the dNTP, or optionally dNTP $\alpha$ S, monomer units and synthesis of the complementary DNA sequence is completed. During the synthesis, a molecule of pyrophosphate is generated for each monomer unit added to the chain. This variation, 25 referred to as Detection-by-Synthesis, proceeds by the detection of pyrophosphate or dNMP as described in the other embodiments where Idling is established to generate additional amounts of pyrophosphate or dNMP. This embodiment may be particularly useful when the Sample Composition contains a relatively large number of copies of the Target Nucleic Acid. In some embodiments of Detection-by-Synthesis, the DNA 30 Polymerase will have a substantial 3'-5' Proofreading Exonuclease activity. This increases the amount of PPi released during synthesis of complementary DNA, as the

-25-

DNA Polymerase will repeatedly add and excise monomer units, even though true Idling will not take place as all 4 dNTPs are present. In this case, it will not be possible to use dNTP $\alpha$ S monomers, as they will prohibit Idling.

In other Detection-by-Synthesis embodiments, an endonuclease can be 5 included in the DNA Polymerase reagent. The endonuclease will nick the Nucleic Acid that is extended from the Imprint or Probe Primer. The DNA Polymerase in this embodiment recognizes these nicks, and will begin DNA synthesis at the 3' end of each nick site, displacing any existing DNA as it proceeds. This phenomenon is well known in the art, and is referred to as "nick translation". This will dramatically 10 increase the amount of DNA synthesis per template molecule, as many DNA Polymerase molecules can simultaneously use one template. This will in turn lead to an increase in the amount of PPi liberated.

In many of the foregoing embodiments, the various reactions in the Imprinting and Idling schemes take place on Solid Phase Supports. Many of these embodiments 15 may use the well-known biotin-avidin interaction as a means for attaching the appropriate Nucleic Acid to the Solid Phase Support. For example, if the Nucleic Acid is labeled with one or more biotin groups, then this Nucleic Acid can bind to an avidin or streptavidin-coated bead. However, this is only one of many possible ways known in the art of attaching Nucleic Acids to Solid Phase Supports. For example, 20 methods are well known in the art that can be used to covalently attach a suitably labeled Nucleic Acid to an appropriately functionalized Solid Phase Support. Any method known in the art for attaching a Nucleic Acid to a Solid Support is contemplated in the present invention.

The geometries of the Solid Phase Support can be varied depending on the 25 application. Preferred embodiments use Solid Phase Supports in the form of beads to which the various reagents are added. In the most preferred embodiment, super-paramagnetic beads are used. These beads can be rapidly separated from reagents by the application of a magnetic field. Other Solid Phase Supports contemplated in the subject invention take the form of microfabricated planar surfaces, known in the art as 30 "biochips". The various primers used in this invention can be attached to spatially discrete locations on said biochips, using any of the methods provided in the art. This

can permit the simultaneous detection of multiple Target Nucleic Acids.

It is also possible, using macroporous monolithic media, to construct cartridges upon which the appropriate Nucleic Acids are immobilized. For example, cartridges can be constructed which have Imprint Primers covalently attached.

5 Reagents can be injected in such cartridges, and then eluted by injection of a wash buffer.

The present invention further includes a diagnostic kit for the detection of Target Nucleic Acids in a Sample Composition. The kit includes at least one Probe Primer that is complementary to one or more portions of the Target Nucleic Acid 10 Sequence. The kit may optionally include one or more Imprint Primers that can be used to create an Imprint, if required, of the Target Nucleic Acid. In preferred embodiments, the Imprint and -- if included -- the Probe Primers, are comprised of Nuclease Resistant Nucleic Acid Residues. The Imprint Primers and an aliquot of the Probe Primers are labelled with a tag, such as biotin, that will allow them to attach to 15 a Solid Phase Support. A Solid Phase Support capable of binding to the labelled Probe Primers and Imprint Primers is also included. This preferably comprises streptavidin-conjugated superparamagnetic beads. Further included in the Diagnostic Kit is a solution containing at least one dNTP and at least one DNA Polymerase. The diagnostic kit also includes a PPi Detection Assay or means for detecting the presence 20 of dNMP.

Related embodiments harness the sensitivity of the PPi enzymatic detection systems without the use of an Idling step. In one such embodiment, an Imprint copy of a Target Nucleic Acid is synthesized in the manner described above. Following synthesis and partial purification, one or more Probe Primers are hybridized to the 25 Imprint. Each Probe Primer comprises sequences complementary to the Target Nucleic Acid, and further comprises one or more molecules of a PPi detection system enzyme. The enzyme molecules can be covalently attached to the Probe Primers by any of the methods known in the art. For example, the Probe Primer may comprise one or more molecules of UDP-Glucose Pyrophosphorylase. In preferred 30 embodiments, the Imprint is bound to Solid Phase Supports as described above to facilitate the separation of Probe-Imprint hybrids from the hybridization reaction. The

hybrids formed between the Imprint and the Probe Primer can be detected by incubating the Solid Phase Supports with PPi and the remainder of the reagents required to converted PPi into NADH: namely UDP-Glucose, Phosphoglucomutase, NAD, and Glucose-6-Phosphate Dehydrogenase. The NADH formed in this way may 5 be converted into light using the bacterial luciferase system described above. In all cases, the enzymatic component that is attached to the Probe Primer is not included in the mixture of reagents that are added after Probe Primer hybridization. This technique may be equally well applied to the Target Nucleic Acid in the Sample Composition without first synthesizing an Imprint.

10 A number of basic principles are known to those skilled in the art that facilitate and enable the present invention. Several of these concepts are set forth below.

15 A) Solid-phase linked probes can efficiently (and specifically) capture complementary nucleic acids from solution.

In one preferred embodiment of the present invention, the various primers-- either Imprint Primers or Probe Primers--are attached to a Solid Phase Support (Figure 3). In one preferred embodiment, the Solid Phase Support comprises superparamagnetic beads. However, linking of the primers to a Solid Phase Support is 20 not essential to the performance of the invention. For example, in certain embodiments of the invention, it is not necessary to isolate the hybrids from the Sample Composition in order to detect for PPi or dNMP. In other embodiments of the invention the hybrids formed can be separated from the Sample Composition without prior attachment of the primers to a Solid Phase Support. For example, the primers 25 could contain a reactive moiety that would react to a Solid Phase Support subsequent to hybrid formation.

30 Using immobilized avidin-biotin probes as the solid phase, the literature contains many demonstrations of (1) rapid (about 10 min.), (2) specific (1 sequence from  $10^6$  others) and (3) quantitative recovery of complementary nucleic acids from solution. This complex or hybrid can be extensively washed without loss of bound primers to remove all unbound nucleic acid as well as any other interfering substances

5 In a preferred embodiment, a linker is attached to the 3' end of the primer sequence. A linker may be attached to the primers by any of the methods known to those skilled in the art. The linker may be comprised of a short alkyl chain, a synthetic polymer such as PEG, a peptide or synthetic nucleic acid linker. The linker can serve to help attach the Imprint Primer to the Solid Phase Support, and also serves to provide a physical separation between the Solid Phase Support and the primers, to allow full access to the primers to all of the components of the Sample Composition.

10 B) Solid-phase linked primer-target hybrids are easily engaged by DNA polymerases and can be extended by them.

15 There are no limitations on the ability of a primer-target hybrid to serve as a substrate for DNA Polymerase-catalyzed DNA synthesis incurred by the hybrid being bound to a Solid Phase Support. It is also well known that a Solid Phase Support bound nucleic acid will interact with a large variety of nucleic acid binding proteins with little if any perturbation from its solution-phase behavior (Figure 4).

20 In one embodiment of the invention, the hybrid is cleaved from the Solid Phase Support before establishing Idling conditions. This situation may be desirable in certain cases where the rate of Idling can be enhanced by allowing the hybrid to exist in solution. Of course, in some embodiments of the invention, the primer need not be attached to a Solid Phase Support at any time during the performance of the method of this invention.

25 C) The pyrophosphate (PPi) released through DNA polymerase catalyzed dNTP incorporation can be assayed easily and quickly at the 0.1 fmol level using a coupled luminescence assay.

In preferred embodiments, the PPi generated during Idling is detected via the generation of NADH. This assay comprises a coupled enzymatic system that generates light as a product. The coupled reactions are as follows:

30 a)  $dNTP + DNA_n \rightarrow DNA_{n+1} + PPi$   
b)  $PPi + UDP-Glucose \rightarrow UTP + Glucose-1-Phosphate$   
c)  $Glucose-1-Phosphate \rightarrow Glucose-6-Phosphate$

-29-

- d) Glucose-6-Phosphate + NAD  $\rightarrow$  NADH + 6- P-glucolactone
- e) NADH + FMN + H<sup>+</sup>  $\rightarrow$  FMNH<sub>2</sub> + NAD
- f) FMNH<sub>2</sub> + O<sub>2</sub> + R-CHO  $\rightarrow$  FMN +H<sub>2</sub>O + R-COOH + **LIGHT**

wherein a) is catalyzed by a DNA Polymerase, b) is catalyzed by UDP-

5 Glucose Pyrophorylase, c) is catalyzed by Phosphoglucomutase, d) is catalyzed by Glucose-6-Phosphate Dehydrogenase, e) is catalyzed by luminescent bacterial oxidoreductase, and f) is catalysed by a bacterial luciferase.

The NADH generating reactions (a-d) and the luciferase reactions (e-f) can all be done in a one-pot system. In preferred embodiments, the reactions are done at 10 separate times. First, all of the PPi is converted to NADH using the UDP-Glucose Pyrophosphorylase/Phosphoglucomutase/Glucose-6-Phosphate Dehydrogenase enzymes in the presence of UDP-Glucose and NAD. Secondly, the 15 oxidoreductase/luciferase reagents are injected into the NADH generation solution. In addition, the efficiency (photons/PPi-sec) can be tuned by increasing/decreasing reagent concentrations.

In other embodiments, the PPi generated during Idling is detected via an ATP-generating system, such as the ELIDA assay. Such assays also comprise coupled enzyme systems that generate light as a product. The coupled reactions for ELIDA are as follows (APS=adenosine 5' phosphosulfate):

20

DNA Polymerase: dNTP + DNA<sub>n</sub>  $\rightarrow$  DNA<sub>n+1</sub> + PPi

ATP Sulfurylase: PPi + APS  $\rightarrow$  ATP + SO<sub>4</sub><sup>-2</sup>

Luciferase: ATP + Luciferin + O<sub>2</sub>  $\rightarrow$  AMP + PPi + CO<sub>2</sub> + Oxy-luciferin + *light*

25

A related assay use NAD-Pyrophosphorylase to generate ATP from PPi through the following series of coupled reactions:

30

DNA Polymerase: dNTP + DNA<sub>n</sub>  $\rightarrow$  DNA<sub>n+1</sub> + PPi

NAD-Pyrophosphorylase: PPi + NAD<sup>+</sup>  $\rightarrow$  ATP + NMN<sup>+</sup>

Luciferase: ATP + Luciferin + O<sub>2</sub>  $\rightarrow$  AMP + PPi + CO<sub>2</sub> + Oxy-

-30-

luciferin + *light*

(APS = Adenosine 5' phosphosulfate, NAD = Nicotinamide dinucleotide, NMN = Nicotinamide mononucleotide)

5 Several other enzyme systems can convert PPi into ATP, and are contemplated in the subject invention. The enzymes and the reactions they catalyze are depicted below:

Adenylate cyclase: cyclic AMP + PPi → ATP

10 ADP-Glucose Pyrophosphorylase: ADP-Glucose + PPi → ATP + 1-phosphoglucose

Poly (A) Polymerase: Poly (A)<sub>n</sub> + PPi → Poly (A)<sub>n+1</sub> + ATP

One of the critical aspects of the present invention is the ability to reduce the production of background light. It is important, therefore, to utilize reagents that are 15 free of contaminating nucleic acids and PPi. Methods are known to those skilled in the art, to simply remove these compounds from reaction solutions and reagents. For example, DNA Polymerase can be cleansed of contaminating nucleic acids (that may fortuitously form hybrids with the Probe Primer or the Imprint Primer) simply by incubating the enzyme at the reaction temperature in the presence of Mg<sup>++</sup>. One major 20 limitation of the ATP generating systems that are used in conjunction with firefly luciferase is that any dNTP (especially dATP) in the Idling mixture will activate the luciferase system. Therefore, the preferred system described above for converting PPi into NADH, followed by the consumption of NADH by bacterial luciferase to generate light, is far less prone to background artefacts than the ATP generating 25 assays. However, if an ATP generating system is used, it is possible to reduce the level of dNTPs through the use of the hexokinase/glycerokinase system described previously.

D) A single T4 polymerase molecule bound to a single probe-target complex can 30 produce about 10PPi/sec using idling turnover.

Idling is a process whereby a DNA polymerase possessing a strong 3' → 5' Proofreading Exonuclease activity can be made to alternatively incorporate a specific dNTP residue (releasing PPi) at a defined location on the target and then excise the newly incorporated dNMP if the next templated dNTP is absent (Figure 5). Both of 5 these reactions are rapid, around 10 sec<sup>-1</sup>. In preferred embodiments of the invention, establishing an Idling condition is critical to the success of the present invention. Even if only one copy of the Target Nucleic Acid is present in the Sample Composition, after ten minutes of Idling, 5,000 molecules of PPi will be generated. As mentioned above, if ten different Probe Primers are utilized -- complementary to 10 different portions of the Target Nucleic Acid -- the amount of PPi generated can be increased by an order of magnitude. In the preferred embodiment, the DNA Polymerase selected will have strong 3'-5' Exonuclease Activity, be thermostable, and will Idle at high rate of turnover.

15 E) Modern porous monolithic Solid Phase Supports are extremely efficient in their mass transport properties.

In one embodiment of the invention, the primers--either Imprint or Probe Primers--are attached to Macroporous Supports. Use of such supports enhances mass transport between the solution phase and the surface of the support -- where the 20 primers are attached.

The empty space in these macroporous systems consists of channels about 1-10 micrometers wide, the interstitial spaces present in bead based media are absent, and injected molecules are always very close to the solid phase (Figure 6)

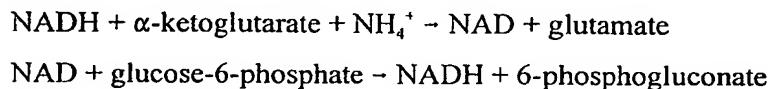
25 F) The amplifications possible by this assay.

Each PPi can in principle be converted to a NADH molecule with 100% efficiency in the presence of an excess of the necessary NADH generating enzymes and reagents. Each of these NADH molecules can be used to activate a luciferase-complex to a state that has a 20% chance of emitting a photon. Thus, a polymerase 30 Idling at the lowest possible rate of 1PPi/second is generating 0.2 photons/second. A single Probe Primer will be the center of generation for the generation of 900 PPi in a

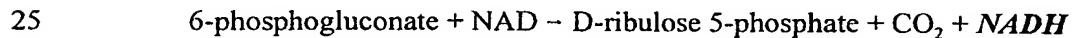
-32-

typical 15 minute Idling reaction. If this PPi is converted to 900 NADH molecules prior to addition of the luciferase cocktail, then addition of the luciferase cocktail will result in the generation of approximately 180 photons. Typical PMT backgrounds are about 1 pulse/sec. Thus, a single hybrid can generate an easily detectable signal. In 5 embodiments where the PPi is used to generate ATP, the same calculation reveals that 15 minutes Idling at the lowest possible rate will provide about 800 photons.

When the PPi is detected by first generating NADH as described above, it is possible to amplify the amount of NADH present in the reaction using coenzyme cycling. One such system is described in Lowry *et al.* 1961. J. Biol. Chem. 10 236:2746, specifically incorporated herein by reference. In this system, the following series of reactions takes place:



15 wherein the reactions are catalysed by glutamate dehydrogenase and glucose-6-phosphate dehydrogenase respectively. NADH is not consumed in these reactions: the net result is the generation of 6-phosphogluconate. After allowing these cycling reactions to proceed for 30 minutes, each NADH molecule from the PPi → NADH reaction scheme gives rise to between 5,000 and 10,000 molecules of 6-phosphogluconate. At the end of the cycling reactions, the enzymes can be heat 20 inactivated. Finally, 6-phosphogluconate dehydrogenase is added, and the following reaction takes place:



Therefore, each NADH molecule in the original solution can give rise to 5,000 to 10,000 NADH molecules in the final amplified mix when the cycling scheme is used. Applying this scheme to the abovementioned calculation for photon emission rates 30 using bacterial luciferase, a single T4 Polymerase Idling for 15 minutes at the lowest possible rate of 1PPi/sec will produce between 900,000 and 1.8 million photons. A

co-enzyme cycling system may be useful in embodiments where the highest possible sensitivity is required.

It is possible to amplify the amount of NADH even further by performing the coenzyme amplification reactions reiteratively, i.e., using the NADH from the third reaction as a substrate in the first reaction. In this way, an initial concentration of NADH as low as  $10^{-16}$  mol can be easily measured with an amplification factor of  $5 \times 10^7$ . The use of reactions that convert PPi to NADH in concert with coenzyme cycling provides a microassay that theoretically has the capability to detect a single PPi molecule. Hence the instant invention easily has the sensitivity to detect a single 10 copy of a Target Nucleic Acid in a Sample Composition.

Further amplifications are possible through the use of multiple Probe Primers. The individual steps in light generation and detection in the NADH system are shown in Figure 7.

15 G) Background Sources

(1) Contamination of the sample with carry over Nucleic Acids.  
(2) PPi present in the dNTP solution (Initially or by spontaneous hydrolysis of the dNTP).  
(3) Interaction of the dNTP solution with Firefly Luciferase at some low level as compared with ATP, but still detectable due to the large amount of dNTP present. This is only a problem in the PPi detection systems that use ATP generation schemes (e.g., ELIDA). The bacterial luciferase system is not activated by dNTPs.

- Source (1) is common to all Nucleic Acid detection technologies and can be minimized by appropriate protocols.  
25 - Source (2) can be minimized by purification of the dNTP solution.  
- Source (3) can be avoided by using the preferred assay system in which PPi is converted to NADH. If the ATP generating schemes are used, it can be minimized by adding a purification step between stages C and D to eliminate dNTP.

The spontaneous hydrolysis rate of the dNTP to form PPi is an impediment to 30 the lowest limits of detection according to this invention.

APPLICATIONS AND EXAMPLES OF THE USE OF THE SUBJECT  
INVENTION

The following examples of applications for the instant method are for illustrative purposes only, and are not meant to limit the scope of the invention.

5

EXAMPLE 1

Detection of a Target Nucleic Acid.

The following procedure illustrates the use of the subject method for detecting a rare Target Nucleic Acid in a Sample Composition. In this example, the method is 10 used to detect the presence of M13 DNA single-stranded DNA in the presence of a very large excess of human genomic DNA. The M13 primer is chosen such that dGTP is the nucleotide that must be added to permit Idling.

The following reagents are used: M13 Solution (0.5 nanomolar M13mp 18(+) strand DNA (Pharmacia) in 50 mM Tris-Cl/1mM EDTA/pH 7.5), Genomic DNA 15 Solution (10 microgram/ml of purified human genomic DNA (Promega) in 50 mM Tris-Cl/1mM EDTA/pH 7.5 passed 50 times through 22 gauge needle to shear DNA), Trap Bead Solution (resuspend 1 ml of Promega Streptavidin MagnaSphere PMP Plus M13 Oligo beads in 1 ml 50 mM Tris-Cl/1mM EDTA/pH 7.5), Capture Solution (2 M NaCl/50 mM Tris-Cl/1 mM EDTA/pH 7.5), Denaturing Solution (1 N NaOH), 20 Neutralization Solution (5 N HCl), Primer Solution (1 micromolar of the -21 M13 sequencing primer (USB) in 50 mM Tris Cl/1 mM EDTA/pH 7.5), Polymerase Mix (2x = 200 mM Tris-Cl pH 8.0, 20 mM MgCl<sub>2</sub>, 10 mM DTT, 2 unit/20 microliter of T4 DNA Polymerase (Boehringer Mannheim), 20 micromolar dGTP (Pharmacia)), and NAD Pyrophosphorylase/Luciferase Mix (2x = 30 micromolar NAD<sup>+</sup> (Sigma), 0.2 25 unit/ml NAD Pyrophosphorylase (Sigma) both in 1:3 dilution of Promega Luciferin/Luciferase ATP Quantitation Mix).

Using the abovementioned reagents, the following was carried out: 6 eppendorf tubes, labeled as follows, were prepared :A=Nothing, B=No primer, C=No M13, D=0 microgram DNA, E=1 microgram DNA, and F=10 microgram DNA. To 30 each tube the following was added: A: 20 microliter Genomic DNA Solution, B: 20 microliter Genomic DNA Solution, C: 20 microliter Genomic DNA Solution, D: 20

-35-

microliter Water, E: 20 microliter 1:10 Genomic DNA Solution, and F: 20 microliter Genomic DNA Solution.

Then, to tubes B, D, E and F, 2 microliter of M13 Solution was added. The tubes A-F were held at 70°C for 5 minutes, then held at 40°C. Next, 20 microliters of the Trap Bead Solution were added to each tube. The tubes were allowed to sit at room temperature for 5 minutes with occasional mixing. The beads in tubes A-F were then magnetically pelleted and washed three times in water. They were resuspended in 10 microliter Denaturing solution, and then magnetically pelleted again. The supernatant was pipetted into new tubes (still labeled A-F) and 2 microliters of Neutralization solution was added to each tube. Next, 1 microliter of Primer solution was added to tubes C, D, E and F. Tubes A-F were allowed to sit at 37°C for 1 minute, and then 10 microliters of Polymerase Mix (2x) was added to each tube and incubated for 5 minutes at room temperature. The reaction was quenched by holding each tube at 95°C for 1 minute. Finally, 20 microliters of NAD Ppase/Luciferase mix (2x) was added to each tube, and the absolute levels of light emission were counted using Bioscan's Lumi-One Luminometer for 10 seconds after allowing 1 minute for the luminescence to come to equilibrium.

The results are illustrated in Figure 8. It can be seen that in Figure 8A that the presence of both M13 template and M13 primer leads to a dramatic increase in the turnover of dGTP to dGMP in comparison to the case where either the M13 primer or the M13 template alone are present. Figure 8B shows the luminescence output. It can be seen that the presence of 100 femtomoles of M13 template can easily be discriminated above the background in a mixture containing up to 10 $\mu$ g of genomic DNA.

25

#### EXAMPLE 2

##### Detection Using an Imprint copy of the Target Nucleic Acid.

###### A) Formation of the Imprint.

30 The Sample Composition is denatured by heating, and biotinylated Imprint Primer is added. The mix is cooled to the polymerization temperature. A DNA

-36-

Polymerase, typically a thermostable DNA Polymerase such as *Taq*, is added along with a suitable buffer, *E. coli* SSB, and phosphorothioate dNTPs (dNTP $\alpha$ S).

Polymerization is allowed to proceed for a few minutes at an appropriate temperature, typically around 70°C.

5

B) Trapping the Imprint and Eliminating non-Imprint Nucleic Acids.

Enough NaCl is added to the Imprint mix above to give a molarity of 2M. Then, avidin-coated paramagnetic beads are added to the mix and allowed to react with gentle shaking for 5 minutes. There is an excess of biotin-binding sites on the 10 beads.

Following incubation, the beads are removed from the mix by the use of a magnet. The beads are washed 3 times in 0.1N NaOH and optionally treated with DNase I or Exo III.

15 C) Hybridization of Probe Primers.

The beads are re-isolated, washed in buffer, and placed in 2M NaCl containing around 1 $\mu$ M of each Probe Primer. The beads are incubated at the desired temperature for a few minutes before removing them from this solution and washing once in TE buffer at 20°C. The temperature chosen for hybridization depends on the 20 melting temperature of the Probe Primer-Imprint interaction.

D) Generating PPI.

The beads are removed from the TE and placed in a DNA Polymerase mix consisting of 10 $\mu$ M of the Idling dNTP, 10nM DNA Polymerase (typically T4 DNA 25 Polymerase, T7:Thioredoxin or the thermostable enzyme from *Pyrococcus woesei*), and the appropriate buffer components. The reaction is allowed to proceed at the DNA Polymerase's optimal Idling temperature for a predetermined amount of time before the reaction is quenched with either heat or EDTA. The supernatant is then removed and used in the next phase.

30

E) Converting PPi to NADH.

The supernatant is adjusted to the following conditions: 50mM Tris-Cl, pH 8.0, 0.1mM EDTA, 1-5mM MgCl<sub>2</sub>, 500μM β-NAD<sup>+</sup>, and 1mM Uridine-5'-diphosphoglucose. The enzymes are then added at the following concentrations:

5 0.25U/ml of uridine-5'-diphosphoglucose pyrophosphatase, 0.4U/ml of phosphoglucomutase, and 0.2U/ml of glucose-6-phosphate dehydrogenase. The reactions are allowed to proceed at 37°C for 5 minutes. The enzymes are then heat killed at 95°C for 3 minutes and the entire mix is spun through a PVDF protein absorbing membrane.

10

F) Converting NADH to light.

The NADH in the mix is quantitated by adding a bacterial luciferase cocktail that is set to deliver a "burst" of light to the mix in front of the luminometer by syringe injection. The cocktail contains oxidoreductase, FMN, decanal, and luciferase in buffer components. For a 2ml reaction volume, the components are added in the following amounts: 0.1mL 0.01% mercaptoethanol, 1.3mL 0.1M sodium phosphate, pH 6.8, 0.2mL 0.42mM FMN, 0.05mL 0.1% decanal in methanol, and 0.2mL 10mg/ml enzyme preparation in distilled water (containing both oxidoreductase and luciferase from *Photobacterium fischerii*; available commercially from Worthington)

20

EXAMPLE 3

A) Using macroporous monoliths, a rapid flow-through hybridization system can be constructed by immobilizing the Primer Probe in the pores of the monolith using a variety of possible chemistries (Figure 9). Thus, cartridges can be assembled which 25 will trap the Target Nucleic Acid by hybridization. The Sample Composition is injected into the column through the inlet, allowed to hybridize for a short time, then expelled through the outlet.

B) Following hybridization of the Target Nucleic Acid, the trap column is 30 exhaustively washed to remove all un-hybridized Nucleic Acid and other, possibly interfering, components from the Sample Composition.

-38-

C) A solution containing T4 DNA polymerase, buffer, and the single dNTP complementary to the next base on the Target Nucleic Acid is added to the column and left to incubate for about 10 min. at 37 C°.

During this time, each polymerase:probe-target complex will generate about  
5 5,000 PPi molecules.

D) The column is emptied into a chamber located next to a photomultiplier tube. To this solution, a second solution is added which contains ATP sulfurylase, adenosine-5' phosphosulfate, Luciferin, Luciferase and a buffer. Each PPi in the  
10 column wash will catalyze the formation of numerous photons, which will be detected by the PMT.

#### EXAMPLE 4

##### Conversion of dNTPs to dNDPs.

15 The supernatant from Example 1 prior to addition of the Luciferin/Luciferase components is adjusted to have 20% glycerol and 5% glucose. 1 unit each of hexokinase and glycerokinase are added and the mix is incubated for 2 minutes at 37°C. The enzymes are then heat killed at 95°C for 3 minutes and the entire mix is spun through a PVDF protein absorbing membrane. NAD to 30μM and 0.2 units of  
20 NAD-pyrophosphorylase are added to the dNTP-free mix. The mix is allowed to incubate at 37°C for 2 minutes. The mix is then heated to 95°C for 1 minute, after which the Luciferin/Luciferase mixture may be added as described in Example 1.

#### EXAMPLE 5

25 Identification of Pathogenic Contaminants in Bodily Fluids, Food Substances, and Environmental Samples.

The method of the instant invention allows for the ultra-sensitive and rapid determination of the presence or absence of any disease-causing or disease-associated nucleic acid in a sample. In cases where it is desired to determine whether a particular  
30 substance is contaminated with a particular pathogen (e.g., HIV, *Salmonella* etc), then the method would make use of an Imprint Primer and a Probe Primer comprised of

sequences that will specifically hybridize to the pathogenic sequences. The method can be easily automated using robotic liquid-handling systems that dispense mixtures of the appropriate reagents into the wells of microtitre plates, wherein each well contains a different sample for testing. This will allow the subject invention to be 5 used routinely in clinical diagnosis applications in which a large number of samples must be assayed.

In other situations, the method may be used to determine the identity of one or more pathogenic contaminants in a sample in which it is known that a contaminant is present, but the identity thereof is unknown. The sample would be contacted with a 10 pool of Imprint and Probe Primers, according to the methods described above, in which each primer is specific for a particular pathogen that may be contained in the sample above. The sequences of the Probe Primers would be chosen such that only one nucleotide need be added for Idling at each Probe Primer hybridization site. Idling conditions would be established, and the presence of PPi would be detected. If 15 PPi is detected, then it is known that one or more of the suspected pathogenic contaminants is contained within the sample. The Imprints would then be isolated from the Idling mixture, and contacted with a subset of the initial pool of primer; Idling conditions would again be established, and if PPi is detected, the process would be repeated with pools of progressively decreasing primer complexity. In this way, it 20 is possible to narrow down and ultimately identify the pathogenic contaminant(s) contained within the sample.

#### EXAMPLE 6

##### Identification of a Target Nucleic Acid, Followed by Sequencing of the Target.

25 In some cases, it will be desirable to first detect the presence of a particular Target Nucleic Acid, and then sequence a further region of the same Target. For example, many viruses are capable of undergoing rapid mutation in selected regions of their genomes, *e.g.*, strain variation in influenza virus and HIV virus. The resulting new variants often have radically different properties, and are resistant to conventional 30 treatments. The current method can be combined with the method of Nyrén, *et al.* (*Anal. Biochem.* 208:171-175 (1993)) and Ronaghi *et al.* (*Anal. Biochem.* 242:84-89

-40-

(1996), specifically incorporated herein by reference) to first detect a viral Nucleic Acid and then rapidly sequence the variable regions thereof without the need to purify the Target Nucleic Acid and perform conventional Sanger sequencing.

What is claimed is:

1. A method for detecting the presence of a specific nucleic acid sequence in a sample composition, comprising:
  - a) introducing a primer complementary to at least a portion of said specific nucleic acid sequence into said sample composition, wherein said primer binds to said specific nucleic acid by hybridization;
  - b) extending said primer by the addition of at least one nucleic acid residue in the presence of a DNA polymerase, wherein pyrophosphate is produced; and
  - c) detecting for the presence of pyrophosphate, wherein the presence of pyrophosphate indicates the presence of said specific nucleic acid in said sample composition.
2. The method of claim 1 wherein said pyrophosphate is produced by the idling of said DNA polymerase.
3. The method of claim 1 wherein said detection comprises the production and detection of NADH, wherein the presence of NADH indicates the presence of said specific nucleic acid in said sample composition.
4. The method of claim 3 wherein the production of NADH comprises:
  - a) reacting said pyrophosphate and uridine-5'-diphosphate-glucose in the presence of uridine-5'-diphosphate-glucose pyrophosphorylase to yield glucose-1-phosphate;
  - b) converting said glucose-1-phosphate to glucose-6-phosphate in the presence of phosphoglucomutase; and
  - c) reacting said glucose-6-phosphate with NAD in the presence of glucose-6-phosphate dehydrogenase, wherein NADH is produced.
5. The method of claim 3 wherein the presence of NADH is detected by a fluorometric assay.

-42-

6. The method of claim 3 wherein the presence of NADH is detected by an electrochemical assay.

7. The method of claim 3 wherein the presence of NADH is detected by a  
5 colorimetric assay.

8. The method of claim 3 wherein the presence of NADH is detected by the generation of light.

10 9. The method of claim 8 wherein said light is generated by reacting NADH in the bacterial luciferase system.

10. The method of claim 9 wherein said bacterial luciferase system comprises an oxidoreductase and a luciferase.

15

11. The method of claim 3 wherein the amount of said NADH is amplified by coenzyme cycling.

20 12. The method of claim 1 wherein said DNA polymerase does not have exonuclease activity.

13. The method of claim 1 wherein said DNA polymerase has exonuclease activity.

25 14. The method of claim 13 wherein said DNA polymerase is selected from the group consisting of T4 DNA Polymerase, T7 DNA Polymerase, T7 DNA Polymerase:Thioredoxin, the thermostable DNA Polymerase from *Pyrococcus woesei*, Klenow Fragment DNA Polymerase, AMV Reverse Transcriptase, and MMLV Reverse Transcriptase.

30

15. The method of claim 1 wherein said primer is attached to a solid support.

-43-

16. The method of claim 15 wherein said solid support is selected from the group consisting of superparamagnetic beads, macroporous monoliths, Langmuir-Bodgett films, functionalized glass, germanium, silicon, PTFE, polystyrene, gallium arsenide, gold, and silver.

5

17. The method of claim 1 wherein said introducing and said extending take place in a single reaction chamber.

18. The method of claim 1 wherein said introducing, said extending and said 10 detecting take place in a single reaction chamber.

19. The method of claim 1 wherein said detecting takes place in a separate reaction chamber from said introducing.

15 20. The method of claim 1 wherein said primer comprises a nucleic acid sequence of from 3-100 nucleic acid residues.

21. The method of claim 20 wherein said nucleic acid sequence comprises a 20 nucleic acid residue.

22. The method of claim 20 wherein said nucleic acid sequence comprises a thermostable nucleic acid residue.

23. The method of claim 1 wherein said detection comprises the production and 25 detection of ATP, wherein the presence of ATP indicates the presence of said specific nucleic acid in said sample composition.

24. The method of claim 23 wherein ATP is produced by the reaction of said 30 pyrophosphate with a compound selected from the group consisting of Adenosine 5' phosphosulfate, nicotinamide dinucleotide, ADP-glucose, cyclic AMP, and poly (A)<sub>n</sub>.

25. The method of claim 23 wherein the presence of ATP is detected by the generation of light.

26. The method of claim 25 wherein produced ATP is reacted with a luciferin and 5 oxygen to generate said light.

27. A method for detecting the presence of a target nucleic acid sequence in a sample composition, the method comprising:

10 a) introducing an imprint primer complementary to at least a portion of said target nucleic acid sequence into said sample composition, wherein said imprint primer and said target nucleic acid bind to each other by hybridization;

b) extending said imprint primer, whereby a copy of said target nucleic acid sequence is produced;

15 c) contacting said copy with a probe primer complementary to at least a portion of said copy, wherein said probe primer and said copy bind to each other by hybridization;

d) extending said probe primer by the addition of at least one nucleic acid residue in the presence of a DNA polymerase, wherein pyrophosphate is produced; and

20 e) detecting for the presence of pyrophosphate, wherein the presence of pyrophosphate indicates the presence of said target nucleic acid in said sample composition.

28. The method of claim 27 wherein said pyrophosphate is produced by the idling 25 of a DNA polymerase.

29. The method of claim 27 wherein said copy is at least partially formed from nuclease resistant nucleic acid residues.

30. 30. The method of claim 27 wherein said copy is at least partially formed from thermostable nucleic acid residues.

-45-

31. The method of claim 27 wherein said imprint primer is at least partially comprised of nuclease resistant nucleic acid residues.
32. The method of claim 27 wherein said imprint primer is at least partially formed from thermostable nucleic acid residues.
33. The method of claim 27 wherein said imprint primer is attached to a solid support.
- 10 34. A diagnostic kit for the detection of a specific nucleic acid sequence in a sample composition, comprising:
  - a) a primer complementary to at least a portion of said specific nucleic acid sequence;
  - b) an extending solution comprising a dNTP and a DNA polymerase; and
  - 15 c) a PPi detection assay.
35. The diagnostic kit of claim 34 wherein said DNA polymerase has exonuclease activity.
- 20 36. The diagnostic kit of claim 35 wherein said DNA polymerase is selected from the group consisting of: T4 DNA Polymerase, T7 DNA Polymerase, T7 DNA Polymerase:Thioredoxin, the thermostable DNA Polymerase from *Pyrococcus woesei*, Klenow Fragment DNA Polymerase, AMV Reverse Transcriptase, and MMLV Reverse Transcriptase.
- 25 37. The diagnostic kit of claim 34 wherein said primers are labeled such that they are capable of binding to a solid phase support.
38. The diagnostic kit of claim 34 wherein said PPi detection assay comprises:
  - i) uridine-5'-diphosphate and NAD;
  - 30 ii) UDP-glucose pyrophosphorylase, phosphoglucomutase, and glucose-6-

phosphate dehydrogenase;

- iii) FMN and a long-chain aldehyde;
- iv) an oxidoreductase and a bacterial luciferase.

5 39. The diagnostic kit of claim 34 wherein said PPi detection assay comprises:

- i) a reagent selected from the group consisting of adenosine 5' phosphosulfate and nicotinamide dinucleotide;
- ii) an enzyme selected from the group consisting of ATP sulfurylase and nictotinamide pyrophosphorylase; and
- 10 iii) luciferin and luciferase.

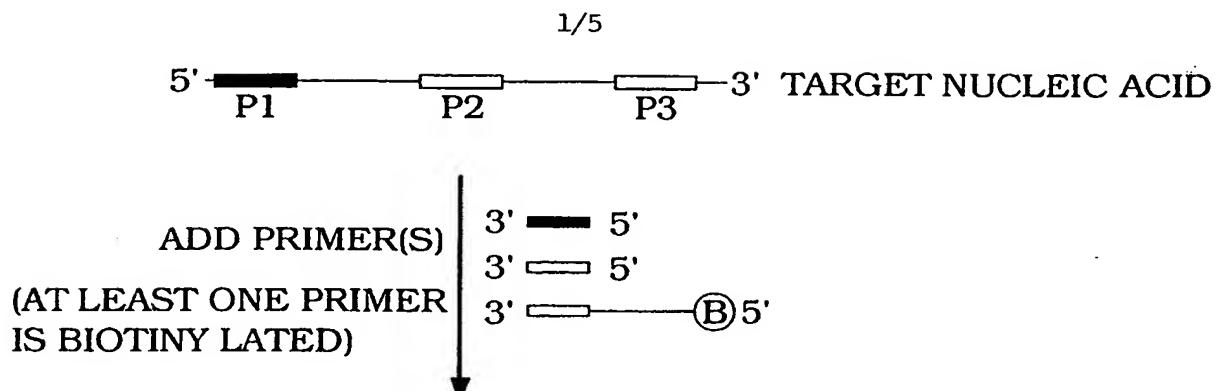


FIG. 1A

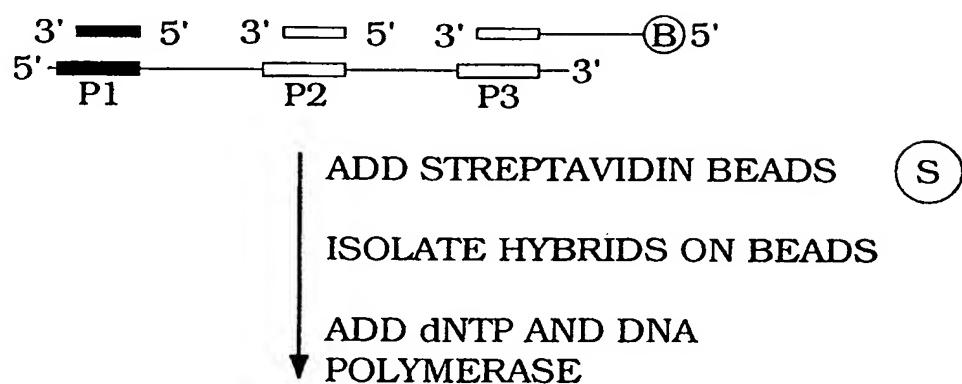


FIG. 1B

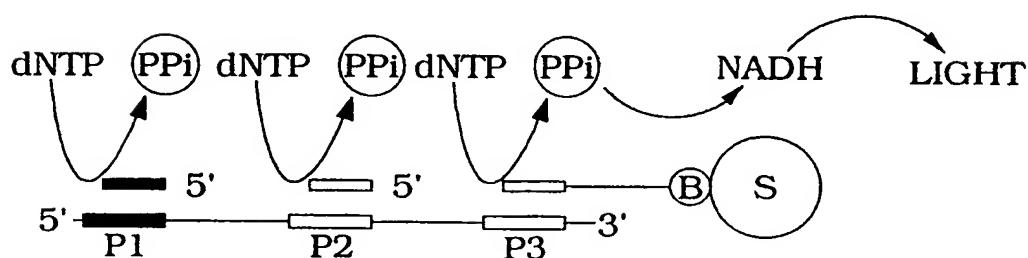


FIG. 1C

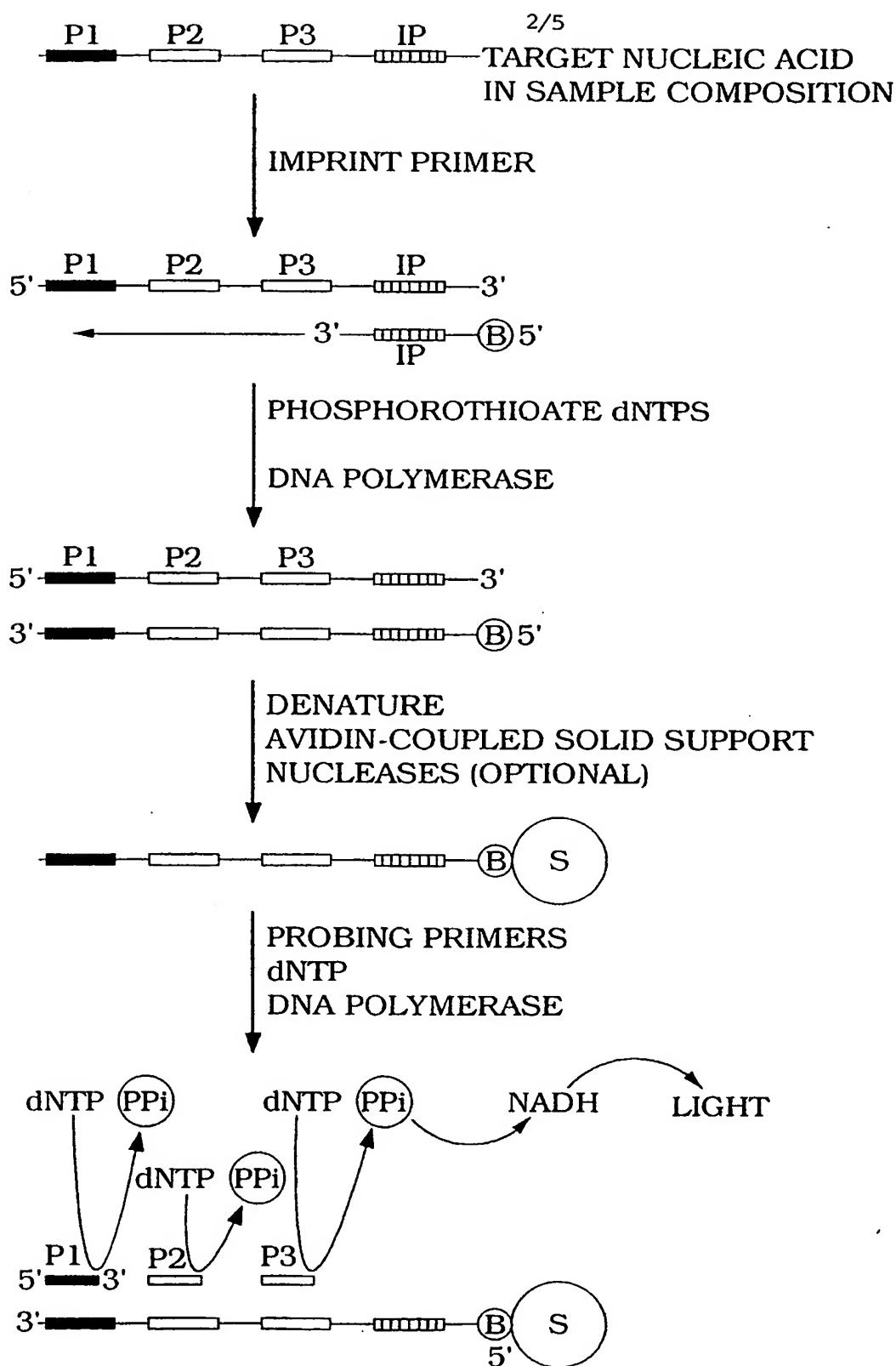


FIG. 2

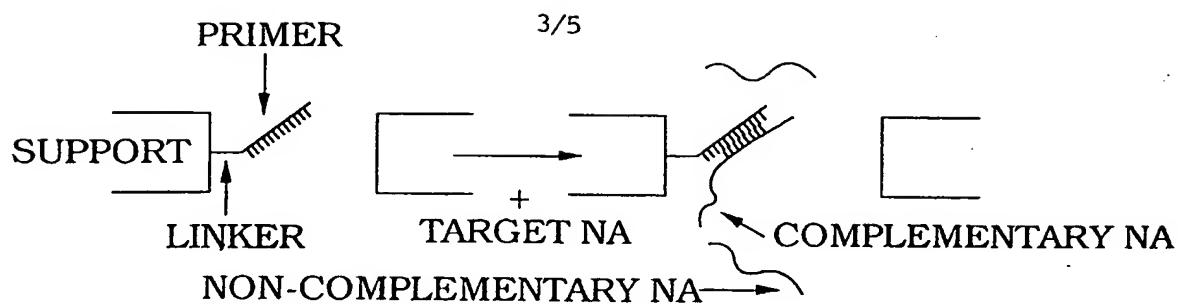


FIG. 3

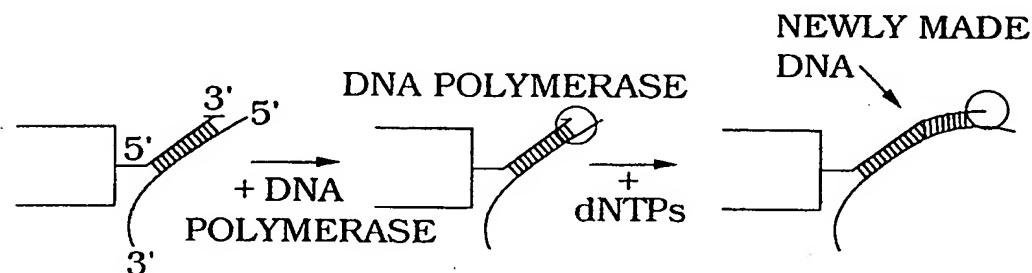


FIG. 4

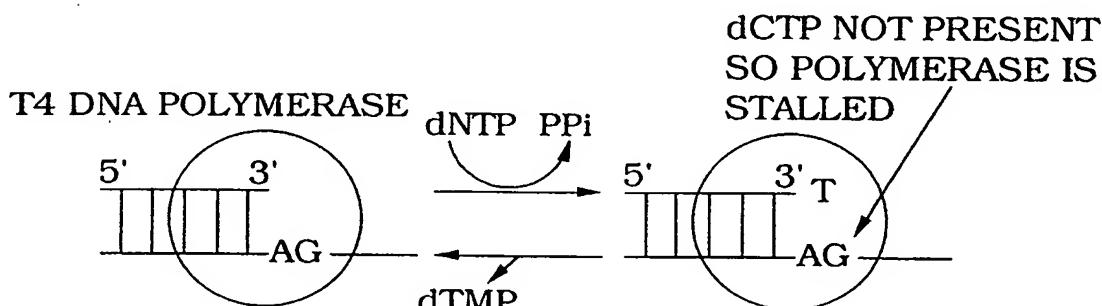


FIG. 5

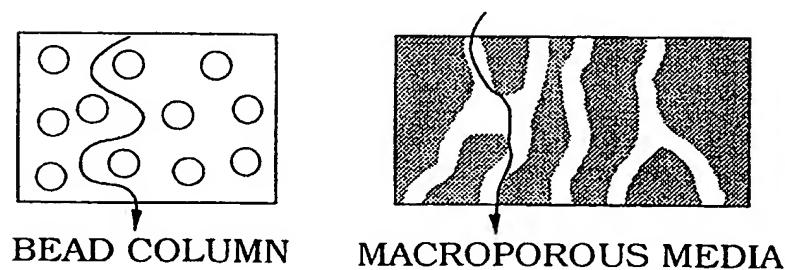


FIG. 6

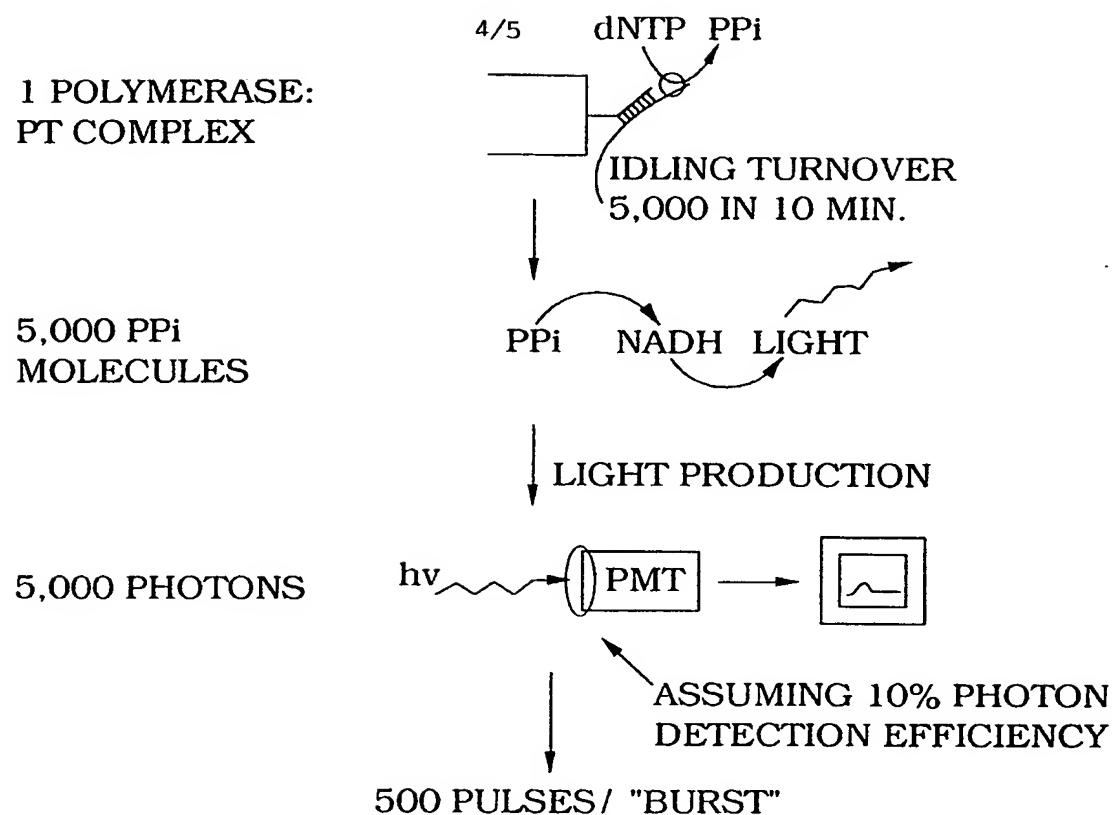


FIG. 7

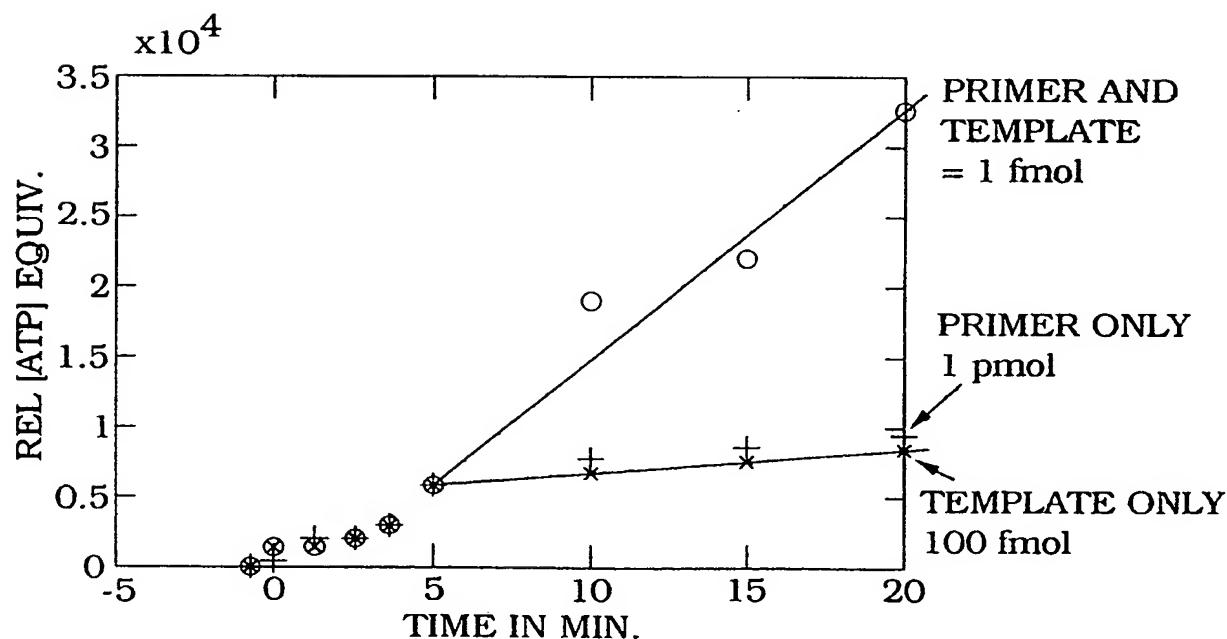


FIG. 8A

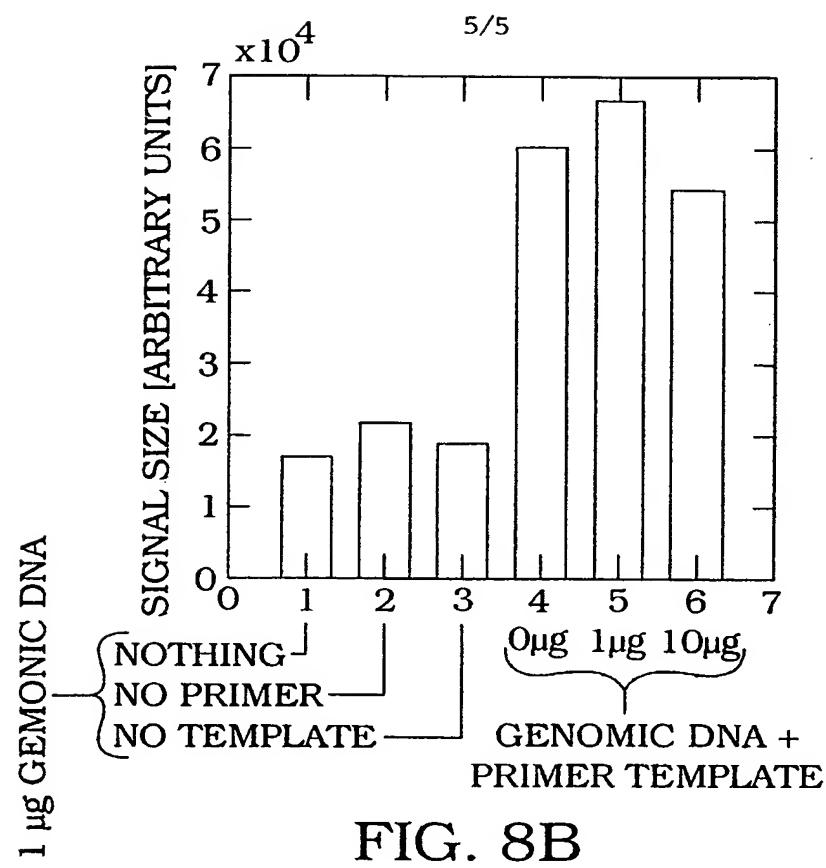


FIG. 8B

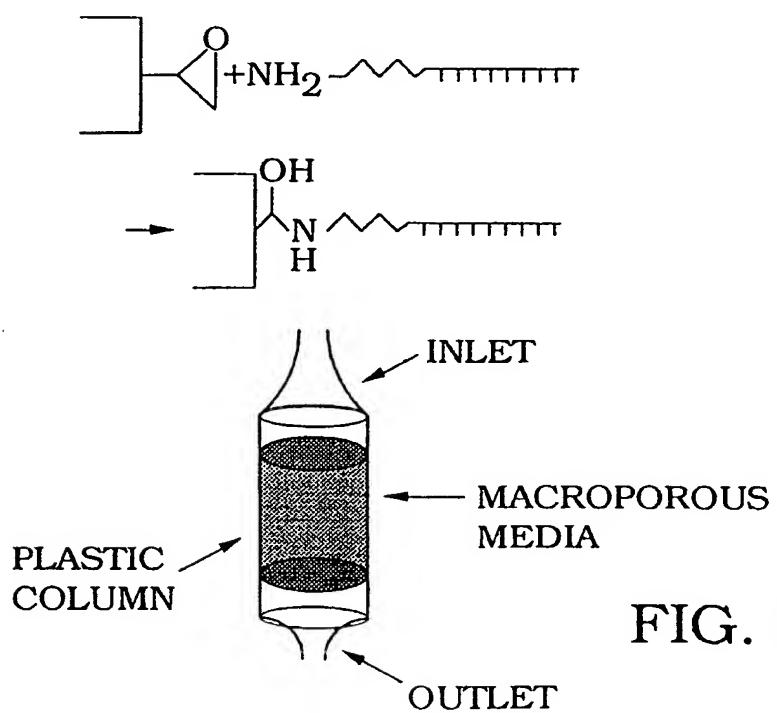


FIG. 9

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/11457

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68

US CL : 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS; BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 362 042 A1 (INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)). 04 April 1990, see entire document.	1-39
Y	US 5,221,736 A (COOLIDGE et al.) 22 June 1993, see entire document.	1-39
Y	US 5,534,424 A (UHLEN et al.) 09 July 1996, see entire document.	1-39

Further documents are listed in the continuation of Box C.

See patent family annex.

Special categories of cited documents:	
"A"	document defining the general state of the art which is not considered to be of particular relevance
"B"	earlier document published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"A."	document member of the same patent family

Date of the actual completion of the international search

13 AUGUST 1998

Date of mailing of the international search report

14 SEP 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

EGGERTON CAMPBELL

Telephone No. (703) 308-0196

